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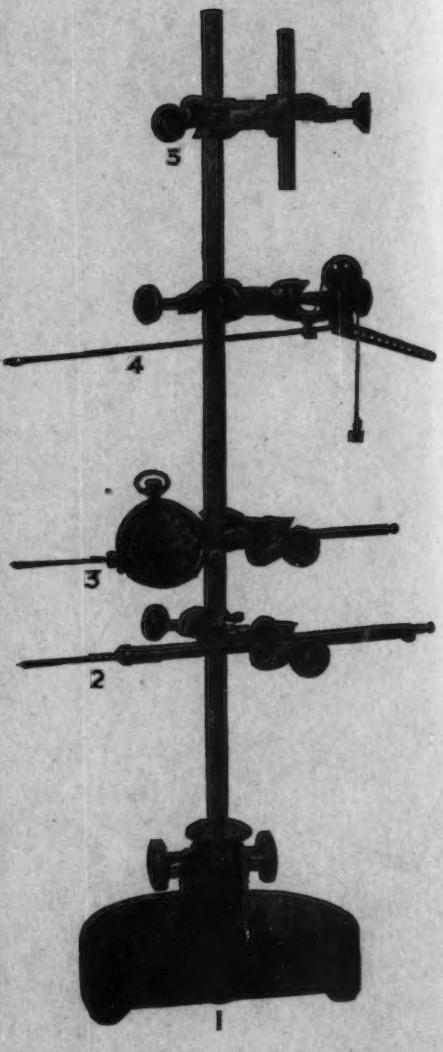

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THE TENSION DEVELOPED BY HUMAN MUSCLES AT DIFFERENT VELOCITIES OF SHORTENING

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In a recent cinematographic study of sprinters it has been shown (Fenn, 1930) that in spite of the difficulty which the muscles experience in shortening rapidly against their own internal viscosity, as pointed out by A. V. Hill (1926), they nevertheless are able to exert enough tension against the rapidly swinging limbs so that 23 per cent of the total energy turnover (oxygen consumption) appears in the limbs as mechanical work. This is a fairly high efficiency and suggests that the importance of viscosity has been somewhat overestimated. There is, however, no doubt that muscles exert less and less tension as their speed of shortening increases. From the point of view of the physiology of running it is of importance to know how much the tension decreases as the speed of shortening increases. Data on this point are available from the work of A. V. Hill (1922) and that of Hansen and Lindhard (1922) using Hill's method. In this case, however, the possibility of reflex inhibition as a contributing cause is not ruled out. Our own measurements show in fact a smaller decrease in tension for the same increase in speed under conditions which apparently make nervous intervention impossible. Even this smaller amount of "viscous loss" may be sufficient to account for a complete loss of external tension in muscles shortening at the speeds which occur in sprint running.

Arm swings. In order to obtain the data needed we adopted a method which has been developed in this laboratory in connection with a study of muscle tonus measurement in man (Smith, Martin, Garvey and Fenn, 1929). Arrangements are made to obtain a kymographic record of the position of a moving arm or leg as a function of time. The slope of this graph gives the velocity of movement at every moment during the movement. If these velocities are plotted out and a smooth graph drawn through the points, then the slope of this curve gives the acceleration of

the movement. Knowing the moment of inertia of the limb, the force exerted at various moments can then be calculated.

In our preliminary experiments the swinging movement of the arm about the shoulder was investigated. The general arrangement is shown in figure 1. The subject leans against one of two upright iron bars with his right shoulder while the other bar is grasped by the left hand. The axis of rotation of the shoulder coincides as nearly as possible with the axis of rotation of the pulley. A wire runs from the pulley to a small pointer sliding on a steel track. The wire is kept taut by a spring. The radius of the wheel was 6.5 cm. so that 6.5 cm. on the drum represented one radian.

Usually we recorded only a single forward swing of the arm extended at the elbow. Sometimes a double swing was recorded both forward and

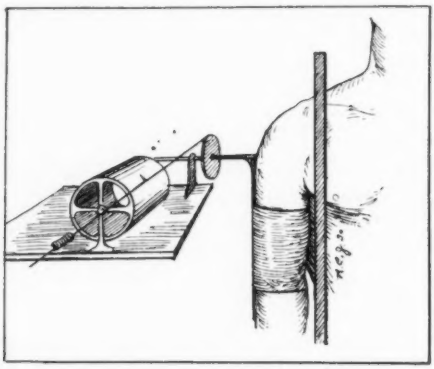

Fig. 1. Diagram of the apparatus used for recording the arm swings

backward as quickly as possible. The results of one such experiment are diagrammed in figure 2. The curve marked y is the displacement curve showing the positions, y, in radians occupied by the arm at various times, t. This is a reproduction of the record as taken on the drum. Measurements were made on the record every 0.01 second. The slope of this displacement curve is given by the curve marked dy/dt and the slope of this angular velocity curve gives in turn the angular acceleration in radians per sec^2 and is marked d^2y/dt^2. Taking 5.8×10^6 gm. cm.^{2} as the moment of inertia of the arm (calculated by method of Braune and Fischer, 1892, or see Fenn, 1930a) the theoretical maximum acceleration can be calculated from measurements of the maximum torque which the subject could exert in different positions of the arm. Thus with the arm extended 60 degrees in front, the subject could pull backwards with a torque of 770 kgm. cm. which corresponds to an acceleration of $770 \div 5.8$ or 136 rad/sec^2. This

value is marked by an arrow at the turning point of the swing (fig. 2) and is seen to coincide with the actual acceleration observed within limits of error of the method.

It is noticeable that the tension does not disappear at the moment of turning, and one of us (W. O. F., 1930) has shown elsewhere that the same

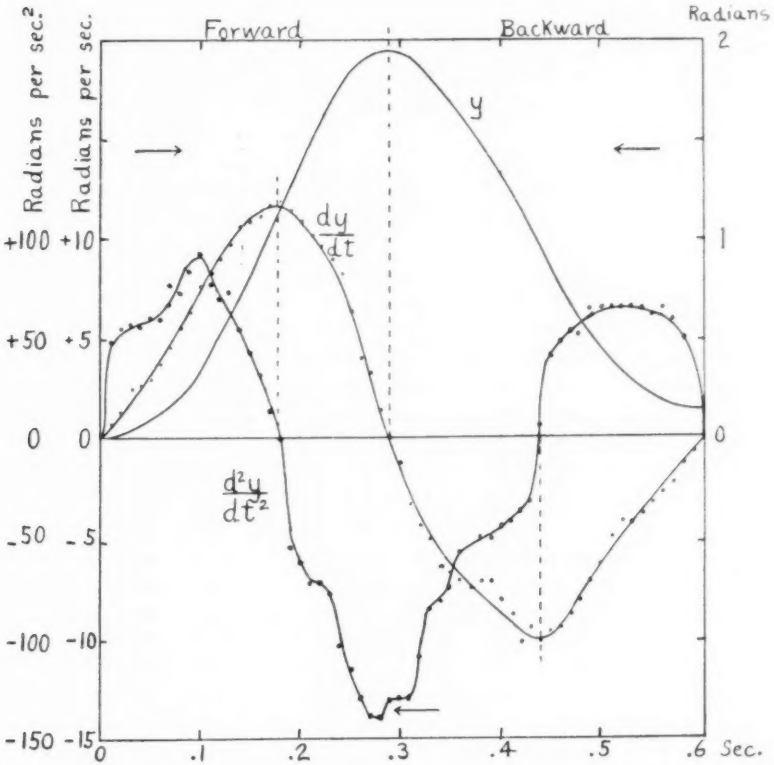

Fig. 2. Graphical representation of the data obtained in one complete swing of the arm forward and backward. See text.

is true for the arm and leg swings which take place during sprinting. It is only at the turning point that the muscles have time to develop their maximum tension against a limb which, for the moment, is not moving. At the beginning of the swing the muscles could not be expected to exert maximum tension for they are trying to keep pace with a rapidly accelerating limb. The experiment shows in fact that the initial acceleration is

only 90 rad/sec² although the muscles are strong enough to have produced an acceleration of 145 rad/sec² if the limb had been stationary or if they had not been engaged in producing energy for shortening at the same time. There seems no reason, however, why the muscles should not have exerted their maximum tension at the end of the swing when they were being simultaneously stretched by the limb which they were trying to decelerate. It may be concluded that the back swing was not carried out with maximum speed and effort.

In most of these preliminary experiments single swings were used. In a few cases a large weight of 3 to 5 kgm. was carried in the hand. Data

TABLE 1

Mechanics of arm movements in subject A. P.

Free swings

NUMBER	AMPLITUDE OF SWING		DURATION		MAXIMUM VELOCITY	WEIGHT CARRIED	MOMENT OF INERTIA	ENERGY		
	Acceleration	Deceleration	Acceleration	Deceleration				Acceleration	K.E. $\frac{1}{2}I\omega^2$	Deceleration
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	degrees	*degrees*	*sec.*	*sec.*	*rad/sec.*	*kgm.*	*gm. cm.²* $\times 10^6$	*kgm. cm.*	*kgm. cm.*	*kgm. cm.*
1	72	89	0.28	0.15	12.0	0	5.8	352	461	474
2	45	40	0.15	0.14	6.97	0	5.8	183	141	180
3	40	45	0.23	0.23	10.0	0	5.8	301	296	286
4	52	46	0.19	0.15	8.4	0	5.8	222	210	208
5 f	63	49	0.18	0.10	11.8	0	5.8	372	409	365
5 b	55	48	0.16	0.17	10.0	0	5.8	285	396	254
6 f	47	49	0.18	0.10	10.2	0	5.8	280	309	288
6 b	43	48	0.12	0.19	10.0	0	5.8	273	296	261
7 f	51	54	0.18	0.20	8.54	3.05	21.9	775	797	706
7 b	40	38	0.19	0.22	4.85	3.05	21.9	266	258	252
8	57	79	0.20	0.40	6.3	5.15	33.0	679	670	745
Average	52.3	53.2	0.187	0.186	—	—	—	362	386	365

from these experiments are collected together in table 1 which shows some points of interest. The first two columns show the amplitude of the swing in degrees during the period of acceleration and deceleration respectively. The average so obtained for the free swings shows that the swing is evenly divided between these two periods, the arm being accelerated for 52.3 degrees and decelerated for 53.2 degrees. The next two columns show the duration of these two periods, acceleration lasting 0.187 second and deceleration 0.186 second. It is a little surprising to find the swing so symmetrical when the purpose in the mind of the subject is to swing his arm forward as rapidly as possible and to stop it at the "end"

as quickly as possible. In carrying out such a swing one feels as if the arm could be stopped almost instantaneously at any time. Actually the antagonistic muscles are contracted to start the deceleration process much sooner than the subject realizes. The experiment illustrates rather well how the cerebrum can dictate a certain whole movement, the pattern for which seems to exist in the lower coördinating centers which carry out the details of the procedure without conscious participation.

Columns 6, 7 and 8, table 1, show respectively the maximum angular velocity attained in the swing, the weight carried in the hand (if any) and the moment of inertia of the system. The presence of a 5 kgm. weight increases the moment of inertia from 5.8×10^6 to 33×10^6 gm. cm.^{2}

Columns 9, 10 and 11 are of especial interest because of the indications which they provide of the general adequacy of the analysis of the records. If in figure 2 the acceleration had been multiplied by the moment of inertia to get the torque exerted and this had been plotted against the position of the arm, y, in radians, then the graph would have been in reality a length-tension diagram, the area of which would have represented the work done on the limb. By integrating such areas graphically we have determined the work done in accelerating the limb (column 9) and the work done in decelerating it (column 11). This includes, of course, the net work done by all possible forces. Thus the work done by the muscles during acceleration is a little larger than the figure given by the amount of work done in overcoming viscosity; and likewise the work done in deceleration by the muscles is a little less than this figure, by the amount of energy taken out by viscosity. As calculated, however, the work done in acceleration should be equal to the work done in deceleration, and both should be equal to the kinetic energy of the limb at the point of maximum velocity. The latter figure is given in column 10 and is equal to half the product of the figures in column 8 by the square of the figures in column 6 $= 1/2\ I\omega^2$. There are some fairly large variations in the individual experiments as might be expected from the roundabout analysis necessary to obtain the figures. There does not seem to be, however, any systematic error in the method greater than 6 per cent because the average of all the figures shows that 362 kgm. cm. was expended in accelerating the limb which then had a kinetic energy of 386 kgm. cm. and which was in turn brought to rest by an expenditure of energy of 365 kgm. cm.

Quick releases of the leg. For more precise study the leg was substituted for the arm, thus avoiding disturbances due to bending of the elbow and movements of the shoulder-blades. The subject was seated as shown in figure 3 with his leg hanging over the edge of a chair. He developed nearly maximum tension against a strong isometric lever which recorded its movements on a revolving drum. By pulling out a pin quickly the leg could be suddenly released and would fly out. The subject was instructed

to "carry through" a complete swing after the release in order to avoid too sudden checking of the movement.

In order to magnify the record for greater accuracy the heel was attached directly to a pointer by means of an aluminum rod. Since the aluminum rod was one yard long the movements of the pointer, at least over the first 10 cm. of the drum record, were proportional, with sufficient approximation to the angular movement of the leg. In certain cases corrections were applied for this error but in the experiments here reported it was too small to take note of. The leg was strapped into a boot-like frame with a steel brace in the back, found in the orthopedic shop. This brace provided a

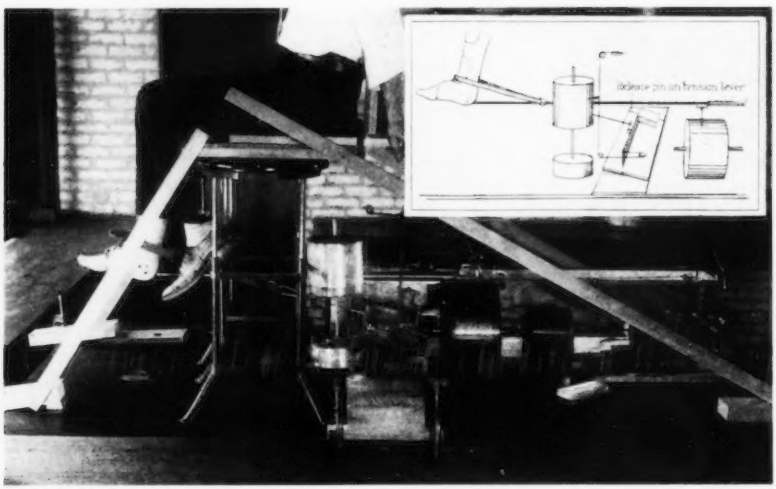

Fig. 3. Photograph and diagram of the apparatus used in quick release experiments.

fixed point of attachment for the aluminum rod. The aluminum rod was fastened at both ends by swivel joints so that there was minimum friction between the carriage bearing the pointer and the track on which it ran. The aluminum rod could not be replaced by a wire for there always seemed to be with this arrangement a slight lag in the start of the pointer so that the recorded acceleration was too great. The drum revolved at a surface speed of 430 cm. per second. The high degree of magnification used in this arrangement makes it impossible to record the complete swing.

One advantage of this method is that errors of recording can be detected with some certainty. Since the moment of inertia of the leg with its attached apparatus is fairly well known, one can calculate the initial accelera-

tion with which it must move from the initial tension developed against the isometric lever at the moment of release. If the method is accurate the

TABLE 2

SUBJECT	NUMBER	INITIAL TORQUE, L	INITIAL ACCELERATION, A	MOMENT OF INERTIA		INITIAL PERIOD				
				Observed L/A	Calculated	Duration	Movement of heel	Velocity attained	Decrease in tension	Slope
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
		dyne cm. $\times 10^6$	*rad/sec²*	*gm. cm.²* $\times 10^6$	*gm. cm.²* $\times 10^6$	*sec.*	*cm.*	*rad/sec.*	*per cent*	
WBL arm	1	406	650	0.625		0.01	4.5	6.0	15.4	2.57
	2	457	820	0.557		0.009	4.0	8.0	22.0	2.75
Average		432		0.59	0.557					2.66
MN leg	3	1,040	190	5.48		0.01	4.0	2.0	10.5	2.25
	4	1,005	190	5.29		0.015	6.0	2.5	5.2	2.08
	5	1,211	225	5.38		0.016	6.5	3.5	11.1	3.17
	6	1,055	205	5.15		0.016	7.8	3.9	8.6	2.53
Average		1,078	203	5.35	5.74					2.51
WBL leg	7	1,210	290	4.17		0.01	4.5	2.7	5.2	1.93
	8	890	250	3.56		0.009	4.0	2.5	18.8	(7.53)
	9	1,210	319	3.79		0.014	5.8	4.0	12.2	3.05
	10	1,210	340	3.56		0.011	4.5	3.4	10.3	3.03
Average		1,130	300	3.77	3.79					2.67
AP leg	11	1,450	370	3.92		0.01	4.4	2.9	10.6	3.65
	12	1,655	370	4.47		0.01	4.6	3.0	10.8	3.60
	13	1,950	390	5.00		0.005	2.2	1.8	36.6	(19.2)
	14	1,950	400	4.88		0.005	1.9	2.0	8.8	4.4
	15	1,618	410	3.95		0.01	4.2	3.6	35.2	(9.8)
Average		1,724	388	4.44	4.8					3.88
Artificial leg				2.96	2.99	Grand average				2.92

Figures in ( ) not included in the average.

Two experiments on the fore arm are included. The upper arm rested on the table the fore arm being held vertical at 90 degrees until released. Otherwise the method was identical with that used for the leg.

observed and calculated values for the acceleration must agree. Conversely, since

$$\text{Moment of inertia} = \frac{\text{torque}}{\text{angular acceleration}} = \frac{L}{A} = I$$

and since L is known from the isometric lever and A can be measured from the record, it is possible to calculate the moment of inertia, I, and to compare it with the estimate made from the length of the leg and the weight of the individual according to the method of Braune and Fischer (1892). In this way the figure of columns 3 and 4 of table 2 were found. Results from 15 quick releases are tabulated. Values are given for L and A and the ratio between them or I. The observed values of I agree within 10 per cent with the calculated values.

In this connection it may also be mentioned that an artificial leg was used in one experiment, strong hammock springs being substituted for

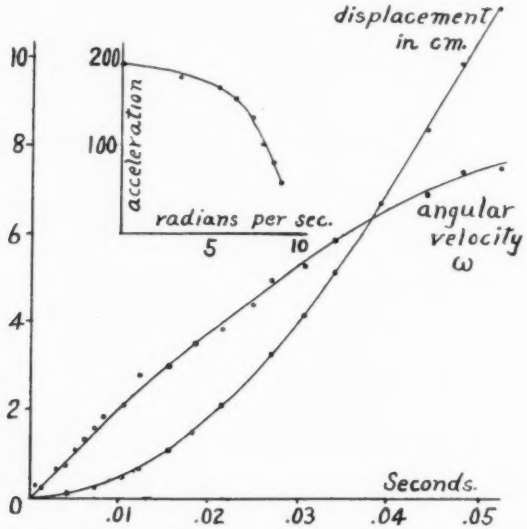

Fig. 4. Graphical representation of an experiment in which the leg was suddenly released at time zero. See text.

muscles. The structure did not pretend to represent a leg in appearance but was simply a hinged affair which happened to be available. Its moment of inertia (2.99×10^6) was determined by the laborious method of taking it all to pieces, weighing the pieces, determining their distances from the axis of rotation so as to get the Σmr^2 for all the pieces. The moment of inertia of pieces of geometrical shape was calculated from familiar formulae wherever possible. The tension of the springs was then determined at different positions of the "leg." A quick release experiment was carried out with this "artificial leg" and the accelerations, determined from the graph for various positions of the "leg" during the swing, were compared with the corresponding tensions of the springs as previously

measured. The ratio between tension, L, and acceleration, A, was found constant throughout the swing as it should have been and equal on the average to 2.96×10^6. This ratio, L/A, is of course the moment of inertia and agrees with the value of 2.99×10^6 determined directly. All this merely served to give us some confidence in the method of analysis.

The detailed results of one such quick release experiment with a real leg are plotted in figure 4. A reproduction of the original smoked drum record is given, the displacement in centimeters being plotted against time in seconds. The slope of this curve gives the angular velocity and these values lie along a fairly smooth curve. Slopes were obtained by the use of a front surface reflecting mirror placed vertically across the curve and turned until the reflection of the graph formed a straight line with the graph itself. A line was then drawn along the edge of the mirror on the record with a

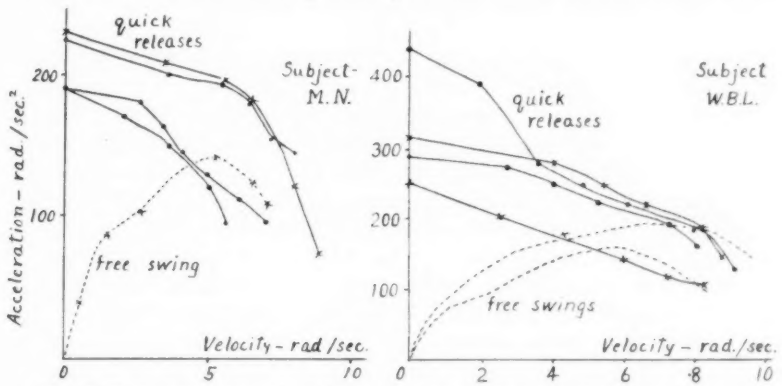

Fig. 5. Experiments on two different subjects showing how the acceleration (or force) decreases as the velocity of the leg increases after a quick release, and how the acceleration increases for a time after the start of a free swing.

sharp instrument and the angle of this line was measured with a protractor. Other analyses were made by the method of subtracting successive ordinates and abscissae and the results were equally good but no better. The initial slope of this angular velocity curve gives the initial angular acceleration from which the moment of inertia is calculated. The results already described in table 2 showed that this initial slope was approximately what it should have been in all cases investigated with the improved method.

The angular acceleration is not plotted against time in figure 4, but it is plotted against the angular velocity, ω, in the insert. This curve is the objective of the whole investigation for it shows the way in which the force (which is proportional to the angular acceleration) falls off as the velocity of the movement increases. The curve falls off rather slowly until

the velocity reaches about 5 radians per second when the fall becomes much more rapid. This rapid decrease may be due to reflex relaxation. The slower decrease which occurs, however, during the first 0.01 or 0.015 second may properly be interpreted as due to processes taking place in the muscles themselves, and may be included under the term muscular "viscosity."

The graphs of figures 5 show the results obtained with two of our subjects. The acceleration (or force) is plotted as ordinates against the angular velocity of the movement. The solid lines represent the quick release experiments while the broken lines are free swings of the leg. Some of the quick release curves are nearly straight lines which would suggest Hill's (1922) interpretation, i.e., that the tension lost was due to a viscosity factor which was proportional to the velocity of the movement. Other curves show distinct breaks suggesting the intervention of some reflex action.

The relation between the force in the free swings and the quick releases is of some interest. The quick release curves supposedly represent the maximum tension which the muscles can exert at the velocities noted. Hence it might be expected that the force in the free swings would gradually increase until the values found in the quick release curve were met with, after which the force in the free swing would have to decrease at the same rate as in the quick release. This is approximately what the curves show.

The purpose of this investigation was to obtain an average figure for the rate of tension loss with increasing velocity of movement. The data for such an average are collected in table 2. The initial tension recorded on the drum by the isometric lever is noted in column 1. Columns 5, 6 and 7 show respectively the duration of the initial period, (preceding the sudden decrease in tension) the extent of movement of the heel during this period, and the velocity attained at the end of this period. Column 8 shows the percentage decrease of the acceleration (and hence of tension) during this period of calculation. The slope of the curve is given in column 9, this being the percentage decrease for an increase in velocity of 1 radian per second. For the subject A. P. who developed the largest initial tension of 1724 dyne cm. $\times 10^6$ the slope was also largest or 3.88. The smallest initial tension was developed by M. N. and the slope calculated from his swings was also the smallest or 2.51 The values for subject W. B. L. are intermediate between these extremes. This correlation with initial tension may possibly be significant, indicating that with high tension the rate of loss is greater. This figure might also mean that the muscles of A. P. were more viscous than those of M. N.

The grand average, from table 2, shows that the tension decreases 2.92 per cent with each radian per second increase in velocity. The maximum duration of the period of measurement is 0.016 sec. and according to

Travis (1928) the latent period for the electrical change of the knee jerk in 43 university freshmen varied from 0.015 to 0.0245 sec. It may be concluded, therefore, that this average slope was measured before any reflex relaxation could have occurred. During this time the heel moves about 4 cm.

In order to get this average slope into more practicable units we may assume that the length of the rectus femoris is 40 cm. and that its lever arm is 3.8 cm. when the knee angle = 90° (cf. K. Fischer, 1927). An angular velocity of 1 rad/sec is then equivalent to a shortening of 3.8/40 or 9.5 per cent of the length of the muscle per second. For a rate of shortening of 10 per of its length per second a muscle will lose, therefore, $2.92 \times \frac{10}{9.5} =$ 3.1 per cent of its tension.

Comparison with the "viscosity constant" of Hill. It has been shown by Hill (1922) that the work, W, done by the flexors of the forearm in contracting from complete extension to complete flexion is given by the formula

TABLE 3

AUTHOR	W_o	k	$\frac{43.5k}{W_o}$	PER CENT TENSION LOST FOR 10 PER CENT SHORTENING PER SECOND
Hill	11.18	2.7	10.5	7.3
Hansen and Lindhard (a)	12	2.35	8.5	5.9
Hansen and Lindhard (b)	14	3.7	11.5	8.0
This paper	—	—	2.92	3.1

$W = W_o - k/t$ where t is the time in seconds occupied by the contraction and W_o is the maximum work in kilogram-meters done in the slowest contraction against the largest equivalent mass. If the total angular movement of the arm was 135 degrees or 2.3 radians then the angular velocity $\omega = 2.3/t$ and the formula may be written

$$W = W_o - k\,\omega/2.3 = W_o - 0.435\,k\omega$$

To make the formula more general, W may be expressed in per cent of W_o, the theoretical maximum work, by multiplying through by $\frac{100}{W_o}$. The formula then becomes.

$$100\,\frac{W}{W_o} = 100 - \frac{43.5\,k\omega}{W_o}$$

In words this means that the muscle loses $43.5\,k/W_o$ per cent of its tension for every increase of one radian per second in the angular velocity of the forearm (see table 3).

Using this formula, numerical values may now be calculated from the data of Hill (1922) and from Hill's treatment (Hill, 1923) of the data of Hansen and Lindhard (1923) for comparison with our own similar constant.

The next to the last column gives the percentage loss of tension for a speed of movement of the limb of 1 radian per second $= \frac{43.5k}{W_o}$. For strict comparability these figures must be corrected for different lengths of muscle and different effective lever arms. The same angular velocity means a much greater rate of shortening in a short muscle with its insertion far from the axis of rotation than the converse. Our figure was corrected on the basis of a lever arm of 3.8 cm. and a muscle length of 40 cm. For the arm muscles a minimum length of 20 cm. may be assumed and a lever arm 2.88 cm.[1] Then an angular velocity of one radian per second is equivalent to a percentage rate of shortening of the muscle of $\frac{2.88}{20} \times 100$ or 14.4 per cent. If, therefore, Hill's figures be multiplied by $\frac{10}{14.4}$ the corrected values of the last column of table 3 are found. The result shows that for a *rate of shortening of 10 per cent of its length per second a muscle loses on the average 7.1 per cent of its tension according to Hill's method and only 3.1 per cent according to our method.*

From this comparison it seems likely that Hill's method includes some nervous inhibition at high speeds which makes the tension fall off more rapidly than it would for reasons of viscosity alone. He has himself suggested such a possibility to explain why his values for W_o were smaller than those obtained by Hansen and Lindhard using a static method.

In comparing these two methods of measuring the viscosity constant of muscles it should be mentioned that the speed of movement in Hill's method is not constant but varies from zero to some maximum during each pull. Only the average speed is used in the calculation. Also Hill measures only the external work while our method measures the force exerted against the limb alone.

[1] Braune and Fischer (see R. Fick, 1911, p. 318) have determined the moments of the forces exerted by the various flexors of the forearm in different positions of the arm. In general the moment increases from 1 cm. to 6 cm. as the degree of flexion increases. An average figure for each muscle may be obtained, however, by integrating the curve over all positions of the arm. This procedure gives the following results: brachioradialis, 5.3 cm., biceps, 3.25, brachialis, 2.4, extensor carpi radialis, 1.88, and pronator teres, 0.9 cm. A weighted average of these figures may be obtained by taking into consideration the contribution to the maximum work made by each of these muscles as given by R. Fick (p. 320). The average so obtained is 2.88 cm. The values lies between that of the biceps and the brachialis which account respectively for 3.84 and 4.85 kgm. m. out of the total of 13.96 kgm. m., and is therefore a reasonable figure to use.

Application to sprinters. From the data of Fenn (1930) on sprinters it is possible to calculate the rates of shortening of the rectus femoris and the biceps femoris at each 0.01 second interval during one running cycle. The necessary anatomical data were obtained from K. Fischer (1927). In this calculation it was necessary to take account both of the movement at the knee joint and of the movement at the hip since both these muscles are two joint muscles. When this was done it was found that the maximum rate of shortening of the rectus femoris was 361 per cent of its length per second, this velocity being reached during the forward swing of the leg. The maximum rate of shortening of the biceps femoris occurs during the back swing just before the foot leaves the ground and it amounts to 373 per cent of its length per second. For the rectus femoris the loss of tension due to viscosity at this rate of shortening would amount to $361/10 \times 3.1$ or 112 per cent; and for the biceps femoris $373/10 \times 3.1$ or 115 per cent. The result shows that neither muscle would be able to exert any external tension at this rate of shortening.

This procedure of course amounts to a very lengthy extrapolation from low velocities to high velocities and it is quite possible that the true relation between tension and velocity of shortening is not a straight line over its whole range. In isolated muscles the tension does in fact tend to fall off less rapidly at the higher tensions (Gasser and Hill, 1924). This is demonstrated also by Levin and Wyman (1927) and explained as due to an undamped component of the elasticity of the muscle. It is to be regretted that in all the published studies of this sort on isolated muscles the speeds of shortening are given in "arbitrary units" so that it is impossible to deduce from work on isolated muscles at how great speeds this linear extrapolation is justified.

At present the only certain conclusion is that the loss of tension due to viscosity can be no greater than 3.1 per cent for 10 per cent shortening per second, although it might be less. We cannot prove conclusively as yet that at the maximum speeds of shortening in running the muscles would be unable to exert any external tension, although this is probably the case. It might be that the muscle simply stopped pulling at the point of maximum velocity in order to give the antagonistic muscles time to decelerate the limb according to the "pattern" of the run. In other words, we cannot be certain that the absence of tension in muscles at high speeds is entirely an "isolated muscle phenomenon" and not in part a central phenomenon. To the extent that it is independent of nervous connections we cannot be certain as yet to what extent it is due to a mechanical interference with the development of tension (which may be taken as a strict definition of viscosity) and to what extent it is due to a chemical delay in the development of the extra energy which is necessary for shortening of the muscle (Fenn, 1922).

SUMMARY

1. Accurate graphical records have been taken of the swinging of the arm from the shoulder and of the leg from the knee. From the records the mechanical features of the swing can be studied. The velocity of movement is first determined and from the change in the velocity with time the acceleration and hence the force exerted by the muscles at different parts of the swing are deduced.

2. The force exerted by the muscles when the limb is held isometrically is determined at the moment when the limb is suddenly released by pulling a pin. From the resulting acceleration of the limb as deduced from the graphical record of the swing the moment of inertia of the limb is calculated and is found to agree within 10 per cent with the value estimated from the weight of the individual and the dimensions of the limb.

3. If the force exerted in such a "quick release" experiment is plotted against the velocity at different moments during the swing, it is found that the tension decreases as the speed of movement increases. The average rate of decrease of tension so determined is 3.1 per cent for a rate of shortening of 10 per cent of the muscle length per second. It is concluded that this represents a maximum figure for the effect of viscosity alone.

4. A similar constant calculated from the data of Hill gives the larger value of 7.3 per cent, probably because reflex inhibition contributed to the decrease of tension.

5. The maximum speeds of shortening of the leg muscles of sprinters were calculated from moving pictures. Assuming a linear relation between tension loss and speed of shortening it is shown that no external tension could be exerted at maximum speeds.

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THE EFFECT OF DIFFERENT PER CENTS OF PROTEIN IN THE DIET

III. Intake and Expenditure of Energy[1]

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In the first paper of this series (Slonaker, 1931) we gave a detailed description of the plan and procedure of this research. Five groups of rats, designated I, II, III, IV, and V, were fed continuously through several generations on carefully prepared synthetic diets containing 10, 14, 18, 22, and 26 per cent protein respectively. Each diet contained all the known amino acids and vitamins and had an energy value of 3.82 calories per gram. The results of this paper are based on the observations of the ten females in each group that were kept in revolving cages and whose food intake could be accurately determined.

In table 1 we have given the average age of each group when placed in the revolving cages and the average daily food consumption for each of the first six 40-day periods. It is noticed that group I was approximately 20 days older than the other groups. Since there is a gradual increase in the daily food intake during the growing period it would be expected that group I would eat a greater amount of food at the beginning than any of the other groups. This relation was maintained during the first three 40-day periods. After this the average daily food intake was modified by reproduction and wastage. During these first three periods there was, with one exception, a reduction in food intake from group I to group V. This indicates that the higher the per cent of protein in the diet the less was the amount required to satisfy the appetite of the animals. Since a gram of food in each of the groups had an energy value of 3.82 calories the energy intake was in proportion to the number of grams ingested.

What effect did these marked differences in energy intake have on some of the physiological functions in the different groups? In table 2 we have given the effect on growth during the first three 40-day periods. It is seen

[1] This research has been assisted by the Department of Physiology, the National Research Council through its Committee for Research in Problems of Sex, the National Live Stock and Meat Board, the Fleishmann Company and their successors, Standard Brands, Inc., and the coöperation of Albers Bros. Milling Company.

that the per cent gain in body weight was in reverse order to that of the amount of food ingested as shown in table 2. That is, group I with an

TABLE 1

Giving the average age of each group and the average daily food in grams used during each of the first six 40-day periods

GROUP	NUMBER	AVERAGE AGE	40-DAY PERIODS					
			1	2	3	4	5	6
I	10	42	14.04	19.73	23.66	26.21*	31.40*	28.37*
II	10	21	10.45	15.62	19.35	20.58	31.72*	24.57*
III	10	22	11.35	13.93	17.30	20.85	26.27*	21.60
IV	10	22	9.58	11.60	14.74	21.29	26.29*	24.15*
V	10	26	8.38	9.97	13.31	23.91*	29.52*	28.00*

* Food wasted and reproduction.

TABLE 2

Giving the average increase in body weight of each group during the first three 40-day periods

GROUP	NUMBER	AVERAGE FIRST WEIGHT	AVERAGE LAST WEIGHT	NUMBER OF DAYS INTERVENING	GAIN		AVERAGE GAIN PER DAY
					grams	*per cent*	
I	10	86	182	111	96	112	0.86
II	10	84	182	120	98	116	0.82
III	10	53	169	122	116	219	0.95
IV	10	45	150	122	105	234	0.86
V	10	33	134	111	101	306	0.91

TABLE 3

Giving the average total calories ingested and used in activity and loss in feces and urine in each group during the first three 40-day periods

GROUP	NUMBER	CALORIES INGESTED				AVERAGE WEIGHT	ACTIVITY		CALORIES LOST IN FECES	TOTAL CALORIES USED, I.E., LOSS PLUS ACTIVITY	CALORIES LEFT FOR GROWTH AND BASAL METABOLISM
		Fat	Carbohydrate	Protein	Total		Miles ran	Calories			
I	10	1,071	6,827	877	8,775	134	585	1,765	1,316	3,081	5,694
II	10	986	4,973	986	6,945	133	393	1,181	1,031	2,212	4,742
III	10	1,035	4,301	1,171	6,507	111	600	1,453	976	2,429	4,078
IV	10	977	3,294	1,218	5,489	97.5	405	861	823	1,684	3,805
V	10	953	2,610	1,271	4,834	83.5	233	424	725	1,149	3,685

energy intake nearly double that of group V showed a per cent increase in body weight of only a little over one-third that of group V. This indicates

that as the per cent of protein increased there resulted a more rapid growth with a marked decrease in the amount of food ingested. Since growth depends on the protein constituents of the diet it will be interesting to make a comparison of the average protein intake in the different groups during this time.

In table 3 we have given the average total calories ingested during the first 120 days and their distribution in fats, carbohydrates and proteins. This shows that even though group I ate almost twice the amount of food as that consumed by group V, the amount of protein ingested was 877 and 1271 calories respectively. This table also shows that regardless of the amount of food eaten there was an increase in the amount of the protein intake from group I to group V. There is thus established a close correlation between the per cent gain in body weight and the amount of protein ingested during this period of growth.

The amount of energy available for growth depends not only on the amount and quality of the food eaten, but also on the amount of energy used in other physiological functions. One of these functions manifested by the rat, which requires a considerable expenditure of energy, is that of spontaneous running activity. This we were able to measure. In table 3 we have given the average number of miles run by each group during the 120 days referred to above. We (Slonaker, 1927) have previously computed the amount of energy required per gram body weight of the rat to run a mile. This was 0.02181 calorie. Having the average weight and miles run by each group and the energy expended per gram-mile, we were able to compute the total energy used in each group in running activity. This is given in table 3. It is noted that group V was the least active and used the smallest amount of energy in activity. Group III was the most active, but, owing to the lighter weight, used a smaller amount of energy than that of group I.

Not all of the energy ingested is utilized by the rat. According to Mitchell and Carman (1926) about 15 per cent of the gross energy of the food is excreted in the feces and the urine thus leaving 85 per cent of metabolizable energy. A determination of the nitrogen contained in the feces of the different groups was carried on for me by E. E. Snyder, chemist. Using Kjeldahl's method he found that the per cent of nitrogen from groups I to V was 3.065, 3.045, 2.59, 2.59, and 4.01 respectively. These per cents would correspond respectively to approximately 19, 19, 18, 18, and 25 per cent protein. This is considerably higher than the loss cited above. Some of this nitrogen may have been due to intestinal bacteria. There was a great difference in the ease of pulverizing the feces for the nitrogen determination. Those of group I were very difficult to reduce to powder. This difficulty became less as the per cent of protein in the diet increased. The feces of group V were easily pulverized. If we assume that 15 per cent is

the loss, the energy not used by each group is given in table 3. Deducting the amount of energy used in running and lost in the feces from the total intake leaves the amount available for growth and basal metabolism. It is seen that this residual amount was largest in group I and became less with the increase of the per cent of protein in the diet. However, if we compute the amount of protein in each of these groups we find that the protein content from groups I to V was 570, 673, 742, 845, and 969 calories respectively. It seems therefore conclusive that the greater per cent gains in body weight (table 2) exhibited by the groups fed the higher per cents of protein was due not to the amount of energy ingested but to the amount of protein available for growth.

After about 120 days in the cages the conditions described above were greatly modified by some of the animals wasting their food and also by reproductive activities. Some of the animals would paw into the food-boxes and throw out the food as though in search of something more appetizing.

TABLE 4

Giving the average total food fed, wasted and eaten in the different groups

GROUP	NUMBER	AVERAGE DAYS IN CAGE	AVERAGE TOTAL FOOD FED	AVERAGE FOOD WASTED		AVERAGE FOOD EATEN	
				Total	Daily	Total	Daily
			grams	*grams*	*grams*	*grams*	*grams*
I	10	798	20,253	5,593	7.00	14,660	18.36
II	10	918	25,439	7,019	7.65	16,420	17.92
III	10	903	20,484	2,435	2.69	18,049	20.11
IV	10	837	19,717	6,319	7.55	13,398	16.00
V	10	894	25,309	7,484	8.38	17,825	19.94

Often almost as much food would be thus wasted as the animal ate. To determine the wastage papers were placed under the cages on which the food was collected. After a period of time the recovered food was weighed and the average daily wastage computed. In table 4 are given the average total amounts of food fed, wasted, and eaten by each group. It is seen that group III had the smallest wastage and consumed the greatest amount of food. This was followed in descending order by groups V, II, I, and IV. The total amount of food eaten depends partly on the life span. As will be noted, the average number of days the different groups were in the revolving cages and during which the food consumption was being determined varied from 798 to 918 days. The average daily food intake is therefore the best for comparison. From this standpoint the order from greatest to least, was groups III, V, I, II, and IV. This shows that the group fed a diet containing 14 per cent protein not only lived the longest, but accomplished this on almost the lowest energy intake of any of the groups.

It is not so much a question of the amount of food consumed by each of these groups as what effect these different amounts and composition of foods had on the physiological activities and efficiency of the animals and how this energy was expended. It has been previously estimated (Slonaker, 1927) that the energy required for the prenatal development of each young was approximately 80 calories and for the postnatal growth to the

TABLE 5

Giving the amount of food used in reproduction and the amount available for other functions in the different groups

GROUP	NUMBER	AVERAGE TOTAL FOOD EATEN	AVERAGE YOUNG BORN	AVERAGE CALORIES USED IN GESTATION	AVERAGE YOUNG WEANED	AVERAGE CALORIES USED IN LACTATION	AVERAGE FOOD USED IN REPRODUCTION		AVERAGE FOOD USED IN ACTIVITY, GROWTH, LOSS AND BASAL METABOLISM
							Calories	Grams	
		grams							*grams*
I	10	14,660	23.7	1,920	17.8	5,340	7,260	1,900	12,760
II	10	16,420	32.3	2,616	24.3	7,290	9,906	2,595	13,825
III	10	18,049	24.0	1,944	15 2	4,560	6,504	1,704	16,345
IV	10	13,398	12.4	1,004	8.6	2,580	3,584	938	12,460
V	10	17,825	7.0	567	3.7	1,110	1,677	439	17,368

TABLE 6

Giving the average energy intake and its computed distribution in the different groups

GROUP	NUMBER	AVERAGE TOTAL MILES RAN	AVERAGE WEIGHT	AVERAGE TOTAL FOOD EATEN	DISTRIBUTION OF ENERGY				AVERAGE MAXIMAL WEIGHT	CALORIES PER GRAM BODY WEIGHT FOR GROWTH AND BASAL METABOLISM	
					Loss in feces and urine	In running activity	In reproduction	Growth and basal metabolism		Total	Daily
			grams	*calories*	*calories*	*calories*	*calories*	*calories*	*grams*		
I	10	2,622	233	56,000	8,400	13,324	7,260	27,016	288	116	0.145
II	10	2,735	220	62,724	9,409	13,123	9,906	30,286	294	136	0.148
III	10	2,795	231	68,947	10,342	14,081	6,504	38,020	307	164	0.183
IV	10	2,546	206	51,180	7,677	11,439	3,584	28,480	247	138	0.165
V	10	2,683	196	67,891	10,184	11,469	1,677	44,561	253	227	0.254

weaning age 300 calories. Using these data we have computed the average amount of energy used in each of the groups during gestation and lactation. This is given in table 5. After deducting the amount of energy used in reproduction from the total intake there is left the energy available for spontaneous activity, growth, maintenance and loss in feces and urine. These amounts are given in the last column of the table. It is interesting

to note that the effect of these different diets on the reproductive efficiency was, from the greatest to least, groups II, III, I, IV, and V.

In our previous paper (Slonaker, 1931b) dealing with the effect on spontaneous activity we gave the average total miles run by each group. These distances are given in table 6. Making use of the data as previously described in this paper we have computed the average energy expended in each group in running the number of miles indicated. The total energy intake less the sum of the amount lost in feces and urine and the amounts used in activity and reproduction gives the amount available for growth and basal metabolism. These data are given in table 6. It is seen that these amounts from greatest to least were in the order of groups V, III, II, IV, and I. But when the average maximal weights were considered we found an entirely different arrangement. From greatest average weight to the least it was groups III, II, I, V, and IV. This indicates that so far as maximal growth was concerned the animals fed on diets containing 10, 14 and 18 per cent protein were able to use the available energy to a much better advantage. This may possibly have been partly due to a higher B.M.R. in groups IV and V. We have not yet made the tests to see if this is true. They are now being undertaken. Using the average body weight, the average number of calories available for growth and basal metabolism and the average number of days the animals were under observation in the revolving cages (table 4) we were able to compute the total and daily number of calories available for growth and basal metabolism per gram of body weight in each group. These amounts are given in the last two columns of table 6. Here again we find that there was a large amount of available energy in group V which seems to have been poorly used.

SUMMARY

When five groups of rats, I, II, III, IV, and V, composed of 10 females in each group were fed continuously throughout life on diets containing 10, 14, 18, 22, and 26 per cent of protein respectively the following results were obtained.

1. Up to approximately 150 days of age the average daily food consumption from greatest to least was in the order of groups I, II, III, IV, and V.

2. During the first 120 days the average per cent increase in body weight from greatest to least was in the order of groups V, IV, III, II, and I. Group V averaged 306 per cent and I 112 per cent again.

3. During the first 120 days the average spontaneous running activity from greatest to least was in the order of group III, I, IV, II, and V.

4. During the first 120 days the energy available for growth and basal metabolism from greatest to least was in the order of groups I, II, III, IV,

and V. The protein content of this energy was 570, 673, 742, 845, and 969 calories respectively.

5. For the whole life span the average daily food intake in grams from greatest to least was group III, 20.11; V, 19.94; I, 18.36; II, 17.92; IV, 16.00.

6. The average total miles ran was group III, 2795; II, 2735; V, 2683; I, 2622; V, 2546.

7. The average number of miles run daily was group I, 3.28; III, 3.09; II, 3.03; IV, 3.01; V, 3.00.

8. The average life span from longest to shortest was in the order of groups II, III, V, IV, and I.

9. The order of number of young born from greatest to least was groups II, III, I, IV, and V. The number of young nursed and weaned was in the same order.

10. The order of average maximal weight was group III, 307; II, 294; I, 288; V, 253; and IV, 247 grams.

11. These results indicate that a diet containing between 14 and 18 per cent protein would result in greatest efficiency in maximal growth, spontaneous activity, reproduction and life span in the rat. They also show that during the growing period the diets containing the larger per cents of protein produced the greater per cents of gain in body weight.

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THE PHYSIOLOGIC ACTION OF RATTLESNAKE VENOM (CROTALIN)

IX. Activity of Protein Fractions of Crotalin

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For many years the belief has been current that the venom of the rattlesnake contains a number of toxic principles. Each of the physiologic manifestations of crotalin has been attributed to a separate toxin. The hemolysis of the blood, the extensive hemorrhage, the marked destruction of tissues, and the nervous symptoms following rattlesnake bite or the injection of cortalin into animals has been conceived as the work of an independent toxin, respectively, a hemolysin, a hemorrhagin, a cytolysin and a neurotoxin. Marshall and Welker made a careful fractionation of the proteins present in the dried venom. These were injected into pigeons with the result that the globulin fraction proved to be inert while the albumin fraction was found to be highly toxic. Since this work was reported more information has been accumulated on the action of crotalin, making available a variety of test objects for the toxicity of this venom. We were interested to determine if the protein fractions of crotalin would manifest only certain activities of the whole venom, as judged by known physiologic responses; for example: whether a protein fraction could be isolated that would cause the typical swelling of the erythrocytes of the dog without lowering the blood pressure, or vice versa, and whether a fraction could be obtained that would cause maximal contraction of the uterus of the virgin guinea pig, but fail to produce whealing when injected into the skin of human beings. If it were possible to secure fractions that possess such selective action it should be convincing evidence of the multiple nature of this venom.

Methods. With these various problems before us we followed the methods of Marshall and those of Welker in procuring the protein fractions of the dried venom; they successfully precipitated the albumin fraction with strong concentrations of ammonium sulphate, and pointed out that the toxic principle was easily denatured and great losses attended precipitation and reprecipitation; we likewise found this to be the case. The following is a description of a typical experiment.

Two hundred milligrams of dried venom of *Crotalus horridus* were dissolved in 4 cc. of physiologic solution of sodium chloride and centrifuged to free it from solid matter. To recover the globulin fraction the crystal-clear supernatant fluid was decanted and 4 cc. of saturated ammonium sulphate were added drop by drop with thorough mixing after each addition. Turbidity slowly developed. Two days later the mixture was centrifuged at 2,800 revolutions each minute for thirty minutes, after which 0.7 cc. of globulin sediment was deposited but the supernatant fluid remained turbid. The supernatant fluid was then filtered, yielding a crystal-clear filtrate. The globulin precipitate was suspended in 15 cc. of half-saturated ammonium sulphate. A soapy solution resulted and the particles slowly aggregated to form a precipitate. After centrifuging at high speed the globulin precipitate measured 0.25 cc. The supernatant fluid was discarded and the sides of the centrifuge tube were wiped dry. The precipitate was then redissolved in 5 cc. of physiologic solution of sodium chloride and 5 cc. of saturated ammonium sulphate was added slowly. After standing for five days the solution was centrifuged and the supernatant fluid was discarded and the fluid adhering to the sides of the test tube was removed. The precipitate, which undoubtedly contained some ammonium sulphate, was dried at 38°C. for three hours and weighed. The globulin fraction thus recovered weighed 8 mgm.

The albumin fraction was isolated by taking the supernatant fluid (7 cc.) from which the globulin fraction had been precipitated and treating it with 2.3 grams of solid ammonium sulphate which was added in small quantities with gentle shaking. After each addition copious heavy precipitation resulted. After centrifuging, the precipitate occupied 0.5 cc. This was redissolved in 5 cc. of a physiologic solution of sodium chloride and resaturated with solid ammonium sulphate. Five days later the solution was centrifuged and the supernantant fluid was discarded. The albumin precipitate, which undoubtedly contained some ammonium sulphate, thus obtained was dried at 38°C. for three hours, after which it weighed 17 mgm.

The 8 mgm. of residue of globulin were dissolved in 3 cc. of a physiologic solution of sodium chloride. Most of this went into solution promptly but a few flakes were evidently denatured and failed to dissolve. The 17 mgm. of albumin were dissolved in 5 cc. of physiologic solution of sodium chloride. On standing 0.1 cc. of flocculent precipitate settled and could not be dissolved.

Results. We have found that the active principle resides in the albumin fraction which is precipitated by gradually saturating a solution of crotalin with solid ammonium sulphate. The globulin fraction prepared by half saturation with ammonium sulphate, by full saturation with sodium chloride, or by dilution with distilled water, has been consistently inert. The albumin fraction, however, exerted the typical effect on the blood

pressure and the volume of the erythrocytes of the dog. The albumin fraction also produced the typical response in the isolated uterus of the virgin guinea pig and when injected into the skin of a human being.

In order to test the toxicity of the globulin and albumin fractions on the blood pressure, a dog weighing 8 kgm. was etherized and the carotic blood pressure was recorded in the usual manner. Two cubic centimeters of the solution of the globulin fraction were injected intravenously without producing any change in the level of the blood pressure. Immediately before the injection a 15 cc. sample of blood was removed, and immediately after

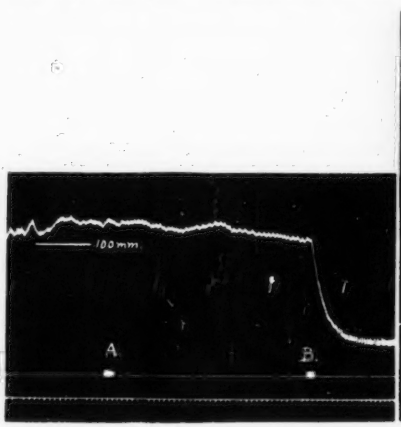

Fig. 1

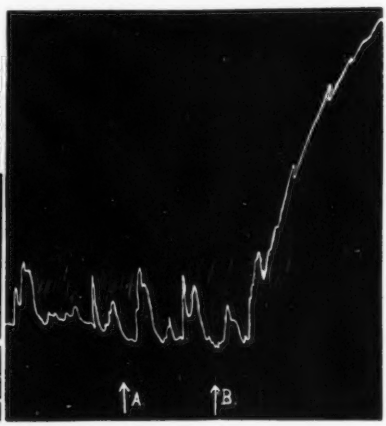

Fig. 2

Fig. 1. This record of the blood pressure of a dog demonstrates the nontoxic effect following the intravenous injection of 2 cc. of the globulin fraction, *A*, as compared with the marked fall resulting from an intravenous injection of 2 cc. of the albumin fraction of crotalin, *B*.

Fig. 2. This record shows the action of the fractions on the isolated uterus of the virgin guinea pig. The addition of 0.5 cc. of the globulin solution to the perfusing fluid, *A*, did not cause contraction but the same amount of the albumin solution, *B*, caused almost immediate maximal contraction.

the injection another 15 cc. sample was removed. After centrifugation, the erythrocyte volume was 5.8 cc. in the first sample and 6.0 cc. in the second. The same procedure was followed with 2 cc. of a solution of the albumin fraction. Following this injection the typical depressor response occurred. The blood pressure fell to 30 mm. of mercury and gradually sank to zero (fig. 1). Accompanying the profound fall in blood pressure urine was voided, as usually occurs in crotalin poisoning. The cell volume for a 15 cc. tube of blood drawn after the injection of the albumin fraction was 10 cc.

The effect of these fractions on the perfused uterus of the virgin guinea pig corresponded to the observations that have been noted. The addition of 0.5 cc. of the globulin fraction to the perfusing fluid failed to produce a response but 0.5 cc. of the albumin fraction caused maximal contraction (fig. 2). When tested by injection into the skin of a human being, the globulin fraction was inert but the albumin fraction caused the typical whealing and arteriolar flare.

The protein-free filtrate from 200 mgm. of rattlesnake venom (containing saturated ammonium sulphate) was on one occasion dialyzed for twenty-four hours against tap water. After dialysis the entire quantity was injected intravenously into a dog with negative results.

COMMENT. The observations of Marshall and Welker have been confirmed, namely, that the toxic fraction of rattlesnake venom is an albumin which can be precipitated by gradual saturation with ammonium sulphate. In agreement with these workers we have found the active principle to be attended with great losses. The globulin fraction, however prepared, was found inert. The presence or absence of peptone in the venom of rattlesnakes is as yet unsettled. It takes several days for saturated ammonium sulphate to precipitate the native proteins of the venom. Obviously if the filtrate is tested before forty-eight hours have elapsed, it will give a positive reaction for biuret. The filtrate, however, continues to deposit precipitate. Besides confirming the work of Marshall and of Welker, our observations show that the albumin fraction alone possesses all the properties of crotalin for which tests have been developed.

SUMMARY

The characteristic activity of rattlesnake venom was found present in the purified albumin fraction of the venom. The globulin fraction was inert as was the protein-free fraction. All efforts to separate the venom into several toxic components were unsuccessful. In the light of present data it must be considered as unproved that the toxic manifestations of crotalin are due to a number of substances.

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THE PHYSIOLOGIC ACTION OF RATTLESNAKE VENOM (CROTALIN)

X. Influence of Crotalin on the Viscosity of Blood

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Early in our experiments (Essex and Markowitz, 1930) with crotalin we were interested in the cause of the marked fall in blood pressure following intravenous injections of small quantities of rattlesnake venom. A series of experiments led to the conclusion that the depressor action of crotalin is due principally to marked peripheral vasodilatation. Since the viscosity of the blood is a factor to be considered when changes in blood pressure occur it was reasoned that decreased viscosity probably accompanied the vasodilatation, thus further decreasing the peripheral resistance. Support was given to this hypothesis by the work of Waud (1927) who found a transient decrease in the viscosity of the blood in anaphylactic, peptone, and histamine shock to which he attributed the accompanying fall in blood pressure. Consequently, the investigations reported here were undertaken. Contrary to our expectations, a tremendous increase in viscosity occurred in blood removed from dogs immediately after injections of crotalin and in blood in which the venom was added in vitro, the increase in viscosity becoming sometimes greater than 300 per cent. Although we have made preliminary determinations on the blood of several animals we shall report at this time only our observations on the blood of the dog.

Methods. Our first determinations were made with a laboratory model Hess viscosimeter, but it soon became apparent that this apparatus was inadequate for our purposes. After a few experiments it was noticed that the changes in viscosity were gradual and therefore should be studied as a function of time. The Hess viscosimeter was quite unsuited to such measurements because of inaccuracies, particularly with high viscosities, and the inability to check readings on a given sample of blood, due to changes of viscosity with time and the rapid settling out of the erythrocytes.

We next utilized the MacMichael viscosimeter, which did not prove satisfactory: first, because 30 cc. of blood were required and it was difficult to keep the blood at a constant temperature, and second, because the

problem of maintaining a uniform suspension of erythrocytes could not be solved to our satisfaction.

Finally the Ostwald viscosity pipette was adopted because it was found to be best suited to the conditions of our experiments. Although this method requires a considerable quantity of blood (7 cc.) it is possible to follow changes of viscosity with time in the same sample. The determination requires on an average only two to three minutes and a uniform suspension of corpuscles is maintained by bubbling air through the pipette between determinations. Also thermostating is easily accomplished by immersion of the pipette in a water thermostat. Duplicate determinations of solutions with constant viscosities check easily within 1 per cent. Whereas this method may not be accurate for determinations of absolute viscosity it is felt that its use here is justifiable since it is capable of revealing at least the relative changes in viscosity of blood with time, following the addition of crotalin.

In our preliminary experiments heparinized blood was used, but it was found that the viscosity of normal heparinized blood increased after standing a few hours. Inasmuch as some of the low concentrations of crotalin caused but slight changes in viscosity, it was necessary to insure constancy of viscosity of normal blood. Defibrinated blood was free of this objection; consequently we made use of defibrinated blood in all subsequent experiments.

In all but one of the experiments reported here only the dried venom of *Crotalus atrox* was used. The potency of this venom was probably as uniform as could be expected. In preparing the solutions of crotalin the following procedure was adhered to rigorously. Seven milligrams of dried *Crotalus atrox* venom were dissolved in 2 cc. of Ringer-Locke solution so that 0.2 cc. of the crotalin solution contained 0.7 mgm. To obtain a 10^{-4} concentration in the blood, 0.2 cc. of the crotalin solution was added to 6.8 cc. of blood, giving a concentration of $\frac{0.7}{10} \times \frac{1}{7} = 10^{-4}$ grams for each cubic centimeter. From the solution of crotalin in Ringer-Locke solution consisting of 7 mgm. for each 2 cc., additional solutions were prepared. To 1 cc. of the crotalin solution 2 cc. of Ringer-Locke solution were added, giving a diluton of $\frac{1}{3} \times 10^{-4}$ grams for each cubic centimeter, to 1 cc. of the latter 2 cc. of Ringer-Locke solution were added, giving a dilution of $\frac{1}{9} \times 10^{-4}$ grams for each cubic centimeter, and so forth. In this way, the blood to be tested was always diluted to the same degree, since 0.2 cc. of the proper venom solution was added as a routine to 6.8 cc. of blood. In obtaining the results with the Ostwald pipette the technic described was rigidly followed.

Results. Although we are primarily concerned with the results obtained by the use of the Ostwald pipette, typical determinations obtained with the Hess and MacMichael viscosimeters are given for comparative

purposes. The effect of a sublethal dose of *Crotalus horridus* venom on the viscosity of the blood was determined by the use of the Hess viscosimeter with the following results:

With the temperature 30°C., and a Hess viscosimeter, the viscosity, relative to water, of normal blood before injection of venom was 6.6, and after intravenous injection of venom it was 9.7. The blood was removed from the dog during the fall in blood pressure and gave an increased viscosity of 47 per cent, as has been indicated.

Readings were made also on the plasma before and after the injection of crotalin. With the temperature 30°C., the viscosity, relative to water, of plasma before injection was 1.85, and after injection was 1.9.

TABLE 1

*Typical set of determinations of viscosity (MacMichael viscosimeter)**

BLOOD	DEFLECTION, DIVISIONS	VISCOSITY RELATIVE TO WATER
Normal	68	4.9
After stirring	79	5.6
After addition and mixing of crotalin (dilution 10^{-4})		
3 minutes	108	7.7
4 minutes	137	9.8
5 minutes†	162	11.6
9 minutes	190	13.6
12 minutes‡	139	9.9
15 minutes	141	10.1
16 minutes	142	10.2

* The temperature was maintained at 38°C.; the reading of deflection for water was 14 divisions.

† After this reading was taken the blood was allowed to remain stationary and then was stirred.

‡ After this reading was taken the blood was stirred.

These results showed increased viscosity in the case of whole blood but there was no appreciable change in the viscosity of the plasma, after the injection of crotalin.

From the foregoing experiment it was evident that the viscosity was decidedly increased when the venom was given in vivo. The results of the next experiment settled the question whether an increase in viscosity occurred when the venom was added in vitro.

The temperature was 25°C., and a Hess viscosimeter was used. The concentration of *Crotalus atrox* venom = 1×10^{-5} grams for each cubic centimeter. The viscosity, relative to water, of normal blood was 4.1; five minutes after adding venom it was 7.0, twenty-five minutes afterward 8.8, and forty-two minutes afterward, 10.2.

After the completion of these experiments it was obvious that increased viscosity invariably resulted when crotalin was added to the blood in vivo or in vitro.

It will be recalled that viscosity is measured in the MacMichael viscosimeter by determining the angular displacement of a suspended disk im-

TABLE 2

*Effect of different concentrations of crotalin (Ostwald viscosity pipette)**

DILUTION 1/243 × 10^{-4} GRAMS IN EACH CUBIC CENTIMETER		DILUTION 1.8 × 10^{-4} GRAMS IN EACH CUBIC CENTIMETER		DILUTION 1.27 × 10^{-4} GRAMS IN EACH CUBIC CENTIMETER	
Viscosity relative to normal blood	Time after mixing blood and venom	Viscosity relative to normal blood	Time after mixing blood and venom	Viscosity relative to normal blood	Time after mixing blood and venom
	minutes		*minutes*		*minutes*
1.00		1.00		1.00	
1.01	4.0	1.00	2.8	1.33	2.7
1.00	6.5	1.02	5.8	1.94	5.2
1.00	8.7	1.31	8.8	2.22	11.3
1.01	13.2	1.46	12.2	2.43	17.2
1.02	15.7	1.65	15.3	2.62	22.0
1.05	21.8	1.78	19.4	2.75	28.9
1.10	26.9	1.89	23.4	2.77	34.9
1.21	31.9	1.93	27.4	2.79	42.9
1.33	36.1	2.00	31.5		
1.45	42.1	2.00	35.5		
1.56	47.1	2.02	40.5		
1.63	52.3	2.07	45.4		
1.68	57.3	2.10	50.6		
1.71	62.3	2.13	55.7		
1.77	67.4	2.15	60.7		
1.81	72.4	2.20	65.6		
1.83	77.4	2.24	70.7		
1.87	82.4	2.27	75.7		
1.89	87.5	2.29	80.8		
1.89	92.5	2.31	85.8		
1.89	97.8	2.30	90.8		
1.91	102.6	2.33	95.8		
1.92	107.5	2.29	100.8		
1.91	112.4				
1.91	117.4				

* The temperature was maintained at 27.5°C.

mersed in a rotating cup which contains the liquid under observation. The viscosity of a liquid relative to water is the ratio of the angular displacement of the disk for the liquid and for water (table 1).

All of the following determinations were made with the Ostwald viscosity pipette. With this instrument the viscosity value is given in terms of the time required for a standard quantity of blood to pass through the capillary

tube when 7 cc. are used in the pipette. In table 2 the time for normal defibrinated blood was ninety seconds and the viscosities relative to this sample of blood are obtained by dividing the time of efflux in seconds by 90. The values of time represent the period in minutes elapsing from the mixing of the blood and venom to the middle of that particular determination. To guard against individual variations in the reaction to crotalin data were obtained by the use of blood from the same dog (table 2).

In table 3 are given the final (maximal) values of viscosity of dog's blood at 27.5°C. with different concentrations of crotalin.

TABLE 3

Maximal values for viscosity with different concentrations of crotalin

CONCENTRATION OF CROTALIN, GRAMS FOR EACH CUBIC CENTIMETER	VISCOSITY RELATIVE TO NORMAL BLOOD*
Normal blood	1.05
$1/243 \times 10^{-4}$	1.93
$1/81 \times 10^{-4}$	2.33
$1/27 \times 10^{-4}$	2.80
$1/9 \times 10^{-4}$	3.17
$1/3 \times 10^{-4}$	3.47
$1/1 \times 10^{-4}$	3.67

* The viscosity of normal fresh blood is taken as 1.00.

COMMENT. When first observed, the striking increase in viscosity was thought to be due to loss of fluids from the circulation and consequent concentration of the erythrocytes. When it was demonstrated that the same result followed whether the venom was added in vivo or in vitro this explanation was untenable. Since the viscosity of the plasma before and after the addition of crotalin remained the same an explanation for the increase was sought in the formed elements of the blood. It was later discovered that the erythrocytes reacted to the venom by decided increases in volume, when the venom was added either in vivo or in vitro. We have no hesitancy in attributing the increased viscosity of the blood, following the addition of crotalin, to the swelling of the erythrocytes. The swelling of the erythrocytes occurs at the expense of the plasma and since the volume of the erythrocytes is greatly increased a higher viscosity logically follows.

It is apparent that the increase in viscosity is a function of time up to a certain limit which appears to be dependent on the integrity of the erythrocytes. Our viscosity determinations clearly indicate that the increase goes on rather gradually up to a certain level, when no further increase is seen. When the erythrocytes have swollen to their fullest extent further increase in viscosity does not occur. At this point hemolysis sets in and the viscosity begins to decrease. When the final values are considered

the rate at which the viscosity increases is roughly directly proportional to the logarithm of concentration of crotalin. It should be pointed out that with weaker concentrations ($1/384 \times 10^{-4}$) a latent period of as much as ten minutes occurs. In higher concentrations it is shorter and in the highest concentrations it is so transient as to be beyond measurement by means of the apparatus used.

A characteristic action of crotalin is its marked depressor effect for which we do not have an adequate explanation. The evidence is substantial that it is due to peripheral vasodilatation but the exact mechanism involved is still an unsettled question. It is hardly necessary to point out that under normal conditions such marked increase in the viscosity of the blood should raise the peripheral resistance, thus causing an increase in blood pressure.

SUMMARY

The addition of crotalin to the blood of the dog in vivo or in vitro causes a marked increase in viscosity which is due to the increase in the volume of the erythrocytes, as the addition of crotalin to the plasma alone does not appreciably change its viscosity.

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THE PROTEIN CONTENT OF MAMMALIAN LYMPH AND THE RELATION OF LYMPH TO TISSUE FLUID

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The literature upon the amount of protein in lymph is summarized in table 1. It is clear that surprisingly high protein concentrations are encountered in lymph from subcutaneous regions, and that the protein in lymph is an extremely variable quantity.

Table 2 represents our own data upon dog lymph. It is of value in displaying protein concentrations in lymph from different regions in the same animal. Again, as in the experience summarized in table 1, one encounters constantly large amounts of protein in lymph, together with great variability in concentration between different areas in the same animal. Several years ago one of us—C. K. D. (1927)—was forced to the conclusion that the capillaries in the skin of the frog were normally somewhat permeable to protein, since frog lymph, as shown in table 1, invariably contains blood proteins. This conception was not in agreement with the classical idea of complete impermeability of the capillaries to blood protein, except in the liver and the intestine. Landis (1927–28) remarks in referring to these observations:

> It seems possible, however, from the recovery described above that the tissue fluid immediately outside the capillary walls can differ widely from the lymph even in the same area. In addition the original filtrate might be modified considerably during its passage through the tissue spaces, the smaller lymph capillaries and lymph glands. Until the absence of these changes is proved it is doubtful whether comparison of lymph and blood plasma can in itself yield definite information concerning the permeability of the capillary wall. Since the capillaries in two situations as different as the mesentery and the kidney appear to be impermeable to protein it seems possible that this may prove to be a general property of the endothelial wall.

Conklin (1930) has shown that the rate of formation of frog lymph is extremely rapid, confirming earlier work by Isayama (1924–25), and that the lymph invariably contains protein. Frogs do not have lymph glands so that these structures can be eliminated as possible participants.

The facts of the situation are that lymph invariably contains protein, and usually in concentrations above 1 per cent. This lymph contains albumin, globulin and fibrinogen. If one assumes, as Landis would have

TABLE 1

Composition of lymph

YEAR	SITE OF COLLECTION	AUTHOR	TOTAL PROTEIN	REMARKS
		Man		
			per cent	
1838	Skin of foot	Marchand and Colberg	0.90	True subcutaneous lymph. No obstruction to veins or lymphatics
1849	Skin of abdomen	Fetzer	4.70	Tumor of anterior abdominal wall opened spontaneously Lymphatic obstruction
1854	Skin lymph, leg	Desjardin	4.28	Tropical elephantiasis. Lymphatic obstruction
1866	Skin of thigh	Dähnhardt	3.37	Elephantiasis following erysipelas. Moderate degree of cardiac decompensation
1871	Tumour of thigh	Gjorgjević	1.30	Lymphangioma with a number of openings. No obstruction
1842	Thoracic duct	Rees	7.08	Thoracic duct from a man immediately after hanging. Fasting
1875	Chylous fistula, prepuce	Hensen	1.70–3.00*	Chylous fistula as a result of tropical elephantiasis
1875	Vulvar elephantiasis	Petters	3.60	Chylous fistula
1890	Thoracic duct fistula	Paton	1.20–1.40	Cachectic patient eating very little food. Starvation lymph
1891	Chylous fistula of thigh	Munk and Rosenstein	3.50, 3.50	Fat could be varied with diet. (1) globulin, 0.7 per cent; albumin, 0.28 per cent. (2) globulin, 1.0 per cent; albumin, 2.5 per cent
1907	Thoracic duct fistula	Sollmann	1.90	Chylous fluid from thoracic duct in neck. Nutrition good

* 19 specimens.

TABLE 1—*Concluded*

YEAR	SITE OF COLLECTION	AUTHOR	TOTAL PROTEIN	REMARKS
		Dog		
			per cent	
1921, 1925	Thoracic duct	Meyer-Bisch et al.	2.90–7.10†	Morphine and ether anesthesia. All animals fasted 24 hours
1927	Thoracic duct	Arnold and Mendel	3.70	Amytal anesthesia
		Horse		
1861	Cervical lymph	Schmidt, C.	3.70	
1874	Loin	Gmelin	2.30	
1874	Head	Leuret and Lassaigne	6.00	
1874	Foot	Geiger	0.66	
1843	Cervical lymph	Nasse	3.90	
1874	Lumbar lymph	Reuss and Emmert	3.00	
1925	Chylous fistula	Zaribnicky	4.30	
		Frog		
1927		Churchill, Nakazawa and Drinker	0.29–0.69	9 animals; curare
			0.74–1.35	38 animals; curare
			1.38–2.17	13 animals; curare
1930		Conklin	0.66–2.00	6 animals; curare

† 21 animals.

us do, that the normal capillary wall, except in liver and intestines, is impermeable to protein, one is confronted with difficulty in explaining the constant presence of protein in lymph; and this constancy is present for all species that have been examined. The alternatives to which one is forced, if one is to adhere to the view that normal capillaries are impermeable to protein, seem to be as follows:

1. The protein in lymph has passed through injured capillary walls. Landis has shown that anoxemia and many other agencies increase capillary permeability. But if some abnormal capillary state is responsible for the universal appearance of protein in lymph from animals apparently wholly normal in every respect, then one is in an awkward predicament when one attempts to define a normal capillary.

2. The protein in lymph is formed by the tissues or by the lymphatic endothelium. It is a tissue or an endothelial secretion. There is no evidence of any sort that blood proteins are manufactured universally. Their formation is usually assigned to the liver, and in the case of fibrinogen the evidence for the site of formation is fairly conclusive. Lymphatic endo-

thelium, except in areas of injury where rapid lymphatic growth is occurring, or in embryos, cannot be shown to be other than entirely inert. The contention that all capillaries are impermeable to protein because urine, cerebrospinal fluid, and aqueous humor are protein free, or practically so, and that consequently the capillaries in the kidney, the choroid and ciliary plexuses are impermeable, fails to take account of the fact that in all three

TABLE 2

Protein in per cent in dog lymph and serum

Refractometric determinations. Lymph taken by cannulas in appropriate vessels. Sodium barbital anesthesia.

DOG	CERVICAL LYMPH			LEG LYMPH	THORACIC DUCT	LEFT ARM LYMPH	KIDNEY LYMPH	BLOOD SERUM
	Right	Left	Average					
2		1.4						
3	3.74	3.57	3.65	5.71				
4		3.21		3.56				
5			4.35	2.29				
6			1.06					
7	1.99	1.99	1.99		2.68			
8	2.47	3.05	2.76					
9	2.42	2.60	2.51		3.66			
10	2.05	2.39	2.22					7.53
11			2.15					6.70
12	1.23	1.67	1.45	1.62				6.34
13	1.24	1.40	1.22					6.79
14			2.15					
15	2.41	1.93	2.67	1.70				
16	1.12	1.32	1.22	0.70				7.96
17			1.84					
18a	2.31	2.13	2.22					
20			2.58					
21			3.38		4.82			8.78
22			2.37		4.47			8.84
23	2.15	2.18	2.17	1.86		2.83	3.79	8.02
24	3.44	4.20	3.80		3.24			8.36
25	1.70	1.62	1.66	1.47	3.17			7.20
26	1.87	2.33	2.10					7.80
27	1.06	1.33	1.20	0.76				

of these instances the capillary endothelium is reënforced by a second layer of cells, either epithelial or endothelial. These three membranes are all specialized structures and do not fall in the category of the ordinary capillaries with a single layer of endothelial cells. It is certain that at least some of the renal capillaries leak protein under normal conditions. Lymph flow from the kidney is surprisingly large (Schmidt and Hayman, 1929–30) and we have found this lymph rich in protein (table 2).

In the place of these two alternatives one may consider that capillaries practically universally leak protein; that this protein does not reënter the blood vessels unless delivered by the lymphatic system; that the filtrate from the blood capillaries to the tissue spaces contains water, salts and sugar in the concentrations found in blood, together with serum globulin, serum albumin, and fibrinogen in low concentration, lower probably than that of tissue fluid or lymph; that water and salts are reabsorbed by the blood vessels and the protein enters the lymphatics together with water and salts in the concentration existing in the tissue fluid at the moment of lymphatic entrance. The lymph from any given drainage area contains a varying amount of protein dependent upon the amount of water absorption which has taken place in the region from which the collection is made, and represents a cross section of the tissue fluid of the area in question.

The conception that lymph and tissue fluid possess an approximate degree of identity depends on a number of observed facts. Most prominent among these is the extraordinary ease with which large molecules and particles enter lymphatics. Modern histology is apparently strongly fortified in the conception of the closed lymphatic capillary against the old idea that lymphatics opened into the tissue spaces. MacCallum (1903) showed that the lymphatics in the dome of the diaphragm did not communicate with the peritoneal cavity. Yet when the diaphragm descended there was a momentary opening which permitted the extraordinarily rapid entrance of particles into lymphatics which has so often been observed in this region. MacCallum pictured an anatomical lymphatic integrity to which he was compelled to add the physiological potentiality of opening when the diaphragm contracted. It requires but little experiment to decide that some similar arrangement may be present in the great subcutaneous region of the body. If one injects a graphite suspension under the skin of the foot of a dog, the draining lymphatic trunk just above the ankle being cannulated, one finds graphite particles in the lymph within five minutes after gentle massage of the skin or mild passive movement. In a similar way milk and red cells can be made to enter lymphatics practically immediately after deposition in the tissues. Histological observation displays closed lymphatics in such regions—closed tubes quite in accord with anatomical tenets. Yet the lymphatics have been entered with the greatest promptitude and blood capillaries in the same region are not. We are inclined to believe that motion or massage, any movement of the tissue in which they exist, causes the lymphatics to lose their anatomical integrity of wall and to become continuous with the tissue spaces. Lymphatic capillaries possess an extreme tenuity of wall and if attached on the outside to surrounding structures might readily be pulled open on motion of the part and yet in entire quiescence exist as closed tubes. In order to explain the ready entrance of particles, of solutions containing protein, etc.,

into the lymphatics, we have thus postulated a state of affairs for lymphatics in general not different from that actually described by MacCallum for the diaphragmatic lymphatics. Under circumstances of absolute quiescence one gets little lymph except from regions of extreme capillary permeability such as the liver. When motion occurs the lymphatics begin to be freely entered by the tissue fluid, and slowly carry away all that is unabsorbable by the blood vessels.

If the conception so given is correct, tissue fluid throughout the body contains protein in concentrations shown for lymph in table 2, and one may well ask what is the function of this extravascular protein. In our opinion two ends are served. First of all the great subcutaneous region in the body is the chief reservoir of readily mobilizable water, and water is held extravascular by the protein in the tissue fluid. Emminghaus (1873) noticed that he could not get reasonable quantities of lymph except from young animals. We have had the same impression and are inclined to believe that capillary permeability is greater in young than in old animals and that more tissue fluid, and consequently more lymph, is found in the young than in the old. Is it not possible that an old man owes his loss in weight, and his dried up appearance, to a progressive decrease in the permeability of his blood capillaries, which is only partially compensated by an increase in capillary blood pressure? Such an individual is perhaps unable to hold water in his tissue spaces because the permeability of his capillaries for protein has in large degree been lost, and he has nothing extravascular to retain water when his capillary blood pressure falls in even slight degree. One may look to some condition such as maintenance of the volume of extravascular water for an explanation of the normal increase of blood pressure in old age, and indeed hypertension in general, and it does not seem extravagant to suggest that decreased capillary permeability, with a consequent lowered supply of extravascular protein, is at the root of the problem.

The second point to be made in connection with the protein in the tissue fluid is the possible relation the degree of capillary permeability expressed by it may have to growth. If one conceives the situation as we have done, that is, the constant transudation through capillaries of a fluid containing protein with eventual removal of the protein by lymphatics, it is clear that if lymphatic obstruction occurs in the presence of a normal blood circulation an abnormally high concentration of tissue fluid protein must result. It is a fact that the group of proliferative changes observed in elephantiasis are based upon lymphatic obstruction, coupled ordinarily with some degree of infection. The latter complication is usual, but not absolutely necessary. In any event it is well known that the prime effect of infection is to increase the leakage of protein from the blood vessels in the region involved, and to add cellular detritus. Again it may be asked whether in the pres-

ence of increased protein concentration in tissue fluid, such as is inevitable with lymphatic obstruction, one does not obtain in the tissue fluid a medium for tissue growth in which first fibroblasts and later more specialized elements begin to flourish.

It would be of great interest to obtain true lymph from cases of elephantiasis. Table 1 contains no safe examples of what is needed. When a fistula occurs the lymph protein may easily fall to normal levels, or little above them, since obstruction no longer exists. Experimental lymphatic obstruction has not been accomplished in a complete and permanent manner, though lymph from the leg of a dog, prepared as described by Reichert (1926) might give indication as to correctness of the view suggested.

SUMMARY

1. Tables are presented showing the protein content of lymph as given in the literature for a variety of animals and as found by the authors in lymph from a variety of regions in the dog.

2. Reasons are given for considering that lymph and tissue fluid are identical.

3. The relations of protein in the tissue fluid to the water content, and to growth, are discussed.

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THE PERMEABILITY OF THE CAPILLARIES OF THE DOG TO PROTEIN

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Starling's (1896) theory of lymph formation assumes a balance between the capillary blood pressure and the colloid osmotic pressure of the blood, which, in turn, is dependent on the impermeability of the capillary wall for protein. This is the theory generally accepted today, and since the time of its elaboration few facts of an essential nature have been added to it. Starling (1894) himself recognized and showed conclusively that there is a difference in the capillary permeability of different organs in mammals. The most permeable are those of the liver, with the capillaries of the intestinal mucous membrane slightly less so. The capillaries of the leg and subcutaneous tissues have been thought to be wholly impermeable or at best only slightly permeable to protein.

Landis (1926) found that the blood pressure in the mesenteric capillaries of the frog varied in the majority of cases between 10 and 14.5 cm. of water. This agreed remarkably well with the osmotic pressure of the plasma colloids as reported by White (1924) who found an average value in 3 cases of 10.3 cm. of water. Furthermore, in his experiments with injected dyes, Landis showed that the rate of diffusion of the dye through the capillary wall was dependent on the capillary pressure rather than on the diameter. At a pressure of 11.5 cm. of water, fluid moved neither in nor out of the capillaries, but when the pressure was increased there was filtration outward and when it was decreased there was absorption. Landis' experiments indicate that the mesenteric capillaries are impermeable to the blood proteins, but in view of other facts should not lead to the generalization that all capillaries are of the same type.

Churchill, Nakazawa and Drinker (1927) believed that the skin capillaries of the frog were normally permeable to protein since the fluid in the lymph spaces always contained from 0.29 to 2.7 per cent of protein. The permeability of the capillary wall to protein would naturally decrease the effectiveness of the colloid osmotic pressure of the blood and filtration of fluid would be increased. The average value for the osmotic pressure of the colloidal proteins of lymph was found to be 42 mm. of water, while that of the blood colloids was 71 mm. of water. The presence

of this protein-containing fluid in the extravascular spaces would decrease the effective osmotic pressure of the blood colloids to only 29 mm. of water. Opposed to this, at least in the frog's mesentery, is a capillary pressure of 122 mm. of water, which would account for the large volume of lymph produced. Conklin (1930) has extended these observations on the skin capillaries of the frog and has shown that foreign proteins, with molecules of varying sizes, injected into the circulation pass easily through the skin capillaries and may be recovered in the lymph in a very short time. This fact she considers evidence for a close relationship and even similarity between tissue fluid and lymph. In no case, however, could she find any absorption by the blood of colloidal molecules injected into the lymph sacs. Thus it would seem that the skin capillaries of the frog are permeable to protein or to colloids in one direction only.

The injection of serum into the subcutaneous tissues in the administration of antiserums is a matter of every-day experience, but the route and forces governing its absorption are far from being understood. Absorption has been ascribed to the lymphatics and to the blood capillaries. Starling (1896) demonstrated the absorption of subcutaneously injected saline by the blood vessels and showed that this was dependent on the osmotic pressure of the plasma proteins. The absorption of serum and of fluids rich in protein, with an osmotic pressure equal to that of blood, cannot be explained as easily. Starling himself suggested that this extravascular protein was taken up by the lymphatics although the evidence was insufficient at that time.

In an attempt to determine more precisely the route and rate of absorption of subcutaneously injected serum, Lewis (1921) injected horse serum subcutaneously into dogs and then by means of a delicate serological test detected its presence in the thoracic duct lymph and in the blood. The serum appeared in the lymph in 40 minutes but was present in the blood only after 3½ hours. Lewis says of this work:

> This experiment indicates that the main route of absorption is lymphatic but that some may take place through the blood vessels. However, it may be that the presence of horse serum in the blood can be explained by some connection between the lymphatics and the blood vessels other than that at the mouth of the thoracic duct.

The presence of the comparatively large right lymphatic trunk was apparently not appreciated by Lewis and since this lymphatic entrance into the blood was not ruled out there is the possibility that the foreign protein might, in this way, have gained entrance into the blood stream. The weight of Lewis' evidence, however, is in favor of direct lymphatic absorption.

Bulloch (1898), who undertook to determine at what periods after the injection of definite numbers of diphtheria antitoxin units the antitoxin

could be demonstrated in the blood of the recipient, found that almost the whole of the diphtheria antitoxin introduced subcutaneously rapidly entered the blood stream. The route of absorption was not indicated by these experiments, but since the antitoxin appeared in the blood within 30 minutes, absorption by the blood vessels was thought to have occurred.

Schulemann (1917) has found that certain colloidal dyes injected subcutaneously into an animal remain at the place of injection for a long period of time, being unable to pass through the capillary endothelial wall.

Shipley and Cunningham (1916), working on the omentum as a factor in absorption from the peritoneal cavity, state that true solutions and pseudo-solutions are absorbed through the blood vessels of the omentum very rapidly. They found that carbon granules were absorbed by the omental vessels, since these were found free in the portal vein and in the livers of animals whose omenta had been withdrawn and floated in solutions containing India ink. They consider it probable that only the very smallest granules are absorbed in this way. This has been confirmed by Poynter (1928), but Higgins and Bain (1930) were not able to demonstrate microscopically either free particles or graphite-laden histocytes in the omental blood vessels following an injection of graphite into an artificially isolated pouch of the omentum.

Batchelder, Field and Drinker (1931), using a colloidal nickel solution, have been able to detect the presence of nickel by a delicate chemical method in the livers of dogs whose omenta were withdrawn and whose right and left lymphatic trunks were completely tied off. Thus it would seem that the capillaries of the omentum are permeable, at least to very small granules.

Krogh (1922), in summarizing the evidence of Lewis and Schulemann, states that colloids in general are unable to diffuse through the normal capillary endothelium. Conklin (1930) has demonstrated a one-way permeability to protein on the part of the skin capillaries of the frog and has suggested that pressure and osmotic relations, both of which normally oppose its return to the blood vessels, may account for the failure of protein to pass from the tissue spaces back through the capillary endothelium.

Because of the scarcity and controversial nature of the existing evidence, it seemed desirable to us to extend a great many of the observations to the capillaries of the dog, studying in particular those regions in which the capillaries are spoken of as normally "impermeable" to protein.

EXPERIMENTAL PROCEDURE. The experiments on capillary permeability and lymphatic permeability were divided into several groups: first, those dealing with the passage of colloids from the blood to the lymph; and second, those dealing with the absorption of colloidal molecules from the subcutaneous tissues and from the peritoneal cavity.

1. *Passage of colloids from the blood to the lymph.* In this series of experiments the dogs were anesthetized with 10 per cent sodium barbital which was given intravenously. The anesthesia was deep and the breathing was usually regular, though in some cases there was obvious movement of the muscles of the neck which must have effectively massaged the subcutaneous lymphatics. Normal sterile horse serum was given intravenously and its presence in the lymph was detected by the use of a precipitin test in which anti-horse rabbit serum was used. Both the right and left cervical lymphatic vessels were cannulated and the lymph was collected from this region. The results in this series were in good agreement. The protocol of a typical experiment is as follows:

Experiment 17. October 24, 1930. Dog. Weight 18.4 kgm.
9:15 a.m. Forty cubic centimeters 10 per cent barbital intravenously.
11:00 a.m. Right and left cervical lymphatic trunks cannulated. No spontaneous lymph flow. Gentle pressure at the base necessary to fill the cannulae.

Rate of appearance of intravenously injected horse serum in cervical lymph

TIME	DILUTIONS OF LYMPH					
	1:10	1:100	1:1,000	1:4,000	1:8,000	1:10,000
Normal lymph	−	−	−	−	−	−
11:30	20.0 cc. normal horse serum given through femoral vein					
11:35 to 12:05	−	−	−	−	−	−
12:05 to 12:35	−	−	−	−	−	−
12:35 to 1:35	+	−	−	−	−	−
1:35 to 2:35	++	+	+	−	−	−
2:35 to 3:00	+++	++	+	+	−	−

This protocol shows that the capillaries of a region spoken of as normally "impermeable" to protein readily permit the passage of protein molecules. The horse serum first appeared in the cervical lymph collected from 1 to 2 hours after the intravenous injection. The amount present gradually increased until, at the end of $3\frac{1}{2}$ hours following the injection, it could be detected in a dilution of 1 part in 10,000. Since the lymph collected at any time cannot represent that being formed at the moment, especially in an animal in which there is no free flow of lymph, it is obvious that the protein molecules must have left the capillaries and even entered the smallest lymphatics some time before they reached the main trunks draining the region.

2. *Passage of colloids from the subcutaneous tissues to the lymph.* A. Absorption of normal serum. The experiments in this series were designed to throw some light on the normal permeability of the subcutaneous lymphatics and blood capillaries, and, if possible, to give an idea of the rate and path of absorption of colloids from the subcutaneous spaces. A

number of experiments were done with remarkably similar results. One typical experiment will be used to illustrate the series.

A dog was anesthetized in the usual way with 10 per cent sodium barbital. The respiration was very quiet and regular during the entire experiment. Both right and left cervical lymphatic vessels were cannulated

TABLE 1

Rate of appearance of subcutaneously injected dog serum in cervical lymph

TIME	PER CENT PROTEIN IN LEFT CERVICAL LYMPH	PER CENT PROTEIN IN RIGHT CERVICAL LYMPH
9:30	1.18	
10:15	1.58	
10:30	1.58	
10:45		1.24
11:00	1.26	1.23
11:40	15.0 cc. normal dog serum infiltrated under skin of neck	
12:00		1.38
12:10	1.78	
12:15		1.32
12:30		1.34
12:40	1.77	
12:45		1.32
1:00		1.33
1:10	1.78	
1:15		1.30
1.30	1.85	
1:45		1.38
2:45		1.48
2:50	1.93	
3:00		1.67
3:10	2.12	
3:15		1.55
3:25	2.19	
3:30		1.70
3:40	2.32	
3:50		1.70
4:00	2.43	
4:10		1.84
4:30	2.58	
4:45		1.17

and there was a slow but steady flow of lymph all day. Fifteen cubic centimeters of normal dog serum with a protein content of 6.9 per cent, determined refractometrically, were infiltrated in three places under the loose skin of the neck without any appreciable distention at the points of injection. In 30 minutes, as shown in table 1, the protein content of the lymph rose and this continued for 5 hours. Throughout the experiment

there was no change in the flow of lymph and so, in the face of the steady rise in the protein content, the possibility of the osmotic withdrawal of water from the lymphatics due to the presence of this extravascular serum, causing only a relative increase in the concentration of the lymph, can be ruled out. Experiments to be cited later, based on the serological detection of serum in the lymph, confirm this. Previous to the injection of serum, the average protein content of the cervical lymph was 1.34 per cent, and at the end of 5 hours it had risen to 2.18 per cent. This experiment clearly shows that extravascular protein enters the lymphatics almost at once. Figure 1 illustrates another experiment of this series in which horse serum was injected subcutaneously.

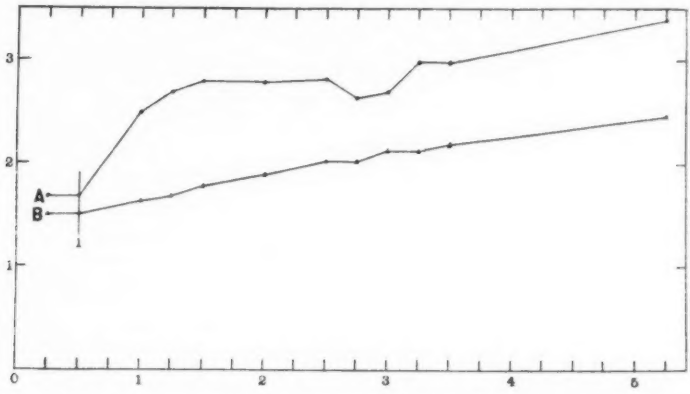

Fig. 1. Rate of appearance of subcutaneously injected horse serum in cervical lymph. *Ordinates*, per cent of protein. *Abscissae*, time in minutes. *A*, left cervical lymph. *B*, right cervical lymph. At mark *1*, 15 cc. normal horse serum were infiltrated under skin of neck.

B. Absorption of foreign serum. Another series of experiments was carried out in which the presence of subcutaneously injected horse serum was detected in the lymph by serological means. The dogs used were prepared in a slightly different way since, at the same time, we wished to determine the permeability of the subcutaneous capillaries for colloidal molecules. The thoracic duct was cannulated and all lymphatics from the head, forelegs, etc., entering the confluence of veins at this place were carefully dissected out and tied off. The large cervical lymphatic was then cannulated close to its entrance into the thoracic duct or into the subclavian vein. The right lymphatic vessel was also isolated and tied off at its venous entrance together with all the other lymphatics of the right side which enter the veins. The cervical branch on this side was also cannulated. At the end of each experiment a very thorough autopsy

of this region was performed to make certain that no lymphatics had been left untied. As a rule the operation was successful, but the great variations which we encountered made this a necessary routine measure. An illustrative experiment of this series is given:

Experiment 19. October 30, 1930. Dog. Weight 18.8 kgm.

10:30 a.m. Fifty cubic centimeters sodium barbital intravenously.

10:45 a.m. to 12:45 p.m. Thoracic duct exposed and cannulated. All tributaries tied off. Right lymphatic and all branches tied at venous entrance. Cannulated.

1:00 p.m. Five cubic centimeters normal horse serum infiltrated under skin of neck, 5.0 cc. under skin of right groin, and 8.0 cc. under skin of anterior abdominal wall. Lymph collections from the thoracic duct and cervical lymphatics made in half-hour or hour periods thereafter for 6½ hours. Horse serum present in cervical lymph in 2 hours and in the thoracic duct lymph in 2½ hours.

Table 2 shows three typical experiments. It is clear from these experiments that at least a great deal of the absorption of serum from the subcutaneous tissues takes place through the lymphatic vessels. The pro-

TABLE 2

NUMBER OF EXPERIMENT	CERVICAL LYMPH UP TO 2 HOURS					CERVICAL LYMPH 2 TO 4 HOURS				
	Dilutions					Dilutions				
	1:10	1:100	1:1,000	1:5,000	1:10,000	1:10	1:100	1:1,000	1:5,000	1:10,000
18a	++	−	−	−	−	++++	+++	+	±	−
19	+	−	−	−	−	+++	++	+	−	−
23	+	−	−	−	−	++	+	−	−	−

tein is taken up rather quickly by the lymphatics and can be detected in the lymph in increasing amounts for 7 to 8 hours. These experiments, also, confirm those in which the estimations of the protein increase in the lymph were made refractometrically, and show that there is a real increase in the protein content rather than a relative one due to the absorption of water from the lymphatics.

It seemed possible that the appearance of the subcutaneously injected serum in the lymph might have been due to the actual puncture of lymphatic vessels by the syringe needle at the time when the injection was made or to the subsequent drainage of the serum into punctured lymphatics. In order to determine just what effect puncturing had, a dog was prepared in the usual way with both cervical lymphatic vessels cannulated. Fifteen cubic centimeters of normal horse serum were infiltrated under the skin of the neck in 6 punctures, several more than usual, and lymph collections were made. A faint trace of horse serum was detected in the lymph in an hour and was present in increasing amounts during the next

four hours. In our other experiments horse serum was very often present in lymph after such an interval of time. Three hours after the first injection, 20 more needle punctures were made in the skin of the neck, but no increased filtration of protein into the lymphatics was observed, and so it is felt that in this and in previous experiments the protein molecules could not have entered the lymphatics through any artificial holes, but must have slowly filtered through the normally permeable lymphatic endothelium.

C. Absorption of fat. One experiment was attempted to determine the path and rate of absorption of olive oil introduced into the subcutaneous spaces. A medium sized dog was anesthetized and the cervical lymphatic vessels were cannulated. The lymph flow was slow but continuous and no massage was employed at any time. Fifteen cubic centimeters of olive oil were infiltrated into the neck and half-hour collections were made. These were extracted with petroleum ether but it was very difficult to tell in this way whether or not fat had been absorbed. Four and one-half hours after the injection of the olive oil the lymph was definitely cloudy or milky in appearance and fat globules were deposited on the inside of the collection tubes. The absorption of fat was undoubtedly more rapid, but our means of detection of small amounts was so poor that we were unable to catch an earlier appearance. There can be no doubt, however, of the slow absorption of fat by the lymphatics. Refractometric determinations of the lymph gradually decreased in value, and since the presence of increasing amounts of fat is known to lower the readings, it was felt that this gave a rough indication of the absorption of fat.

A second experiment was done in which milk stained with Sudan III was used. There was no change in the color or appearance of the lymph for 1 hour and 40 minutes. At the end of this time the neck was massaged gently by stroking for a period of 5 minutes. Following this, the lymph collected was definitely pink in color showing that the stained fat had actually penetrated the lymphatic endothelium.

In another experiment the leg of a dog was infiltrated with milk stained with Sudan III and collections of lymph were made from a cannula inserted into the small groin lymphatic. Continuous massage was applied for 7 minutes following the injection. The lymph collected at the expiration of this time was normal in appearance. Further massage for 5 minutes more caused the lymph to assume a definite pink color, indicating that the milk had entered the lymphatics.

The absorption of extravascular fat is a much slower process than the absorption of serum, though it definitely takes place and can be considerably hastened by massage just as the absorption of protein can be increased in the same way.

3. *Passage of colloids from the peritoneal cavity to the lymphatics.* Ex-

periments were planned to correlate the speed of absorption of serum by the subcutaneous lymphatics with the absorption by those lymphatics of the peritoneal cavity which eventually enter the thoracic duct. The main lymphatic paths of absorption from the peritoneal cavity have been shown by Higgins and Graham (1929) to be via those which enter the right lymphatic duct and the right subclavian confluence of veins. Some absorption, however, takes place through the thoracic duct. Two successful experiments were done. The thoracic duct was cannulated and the presence of horse serum which had previously been injected intraperitoneally was determined by serological means. Table 3 shows the results of a typical experiment. It will be observed that in 30 minutes the protein could be detected in a dilution of 1 part in 400. The lymphatics of the peritoneal cavity, as previous observations have indicated, take up protein even more readily than do those of the cervical region.

TABLE 3

Rate of appearance of intraperitoneally injected protein in thoracic duct lymph

TIME	DILUTIONS				
	1:10	1:50	1:100	1:400	1:800
Control	–	–	–	–	–
1:20	16.0 cc. horse serum injected intraperitoneally				
1:50	+	–	–	–	–
2:20	++	+	–	–	–
2:50	+++	++	++	–	–
3:20	++++	++++	+++	+	–

4. *Passage of colloids from the subcutaneous tissues to the blood capillaries.* The permeability of the normal blood capillaries to protein in regions hitherto spoken of as impermeable has been demonstrated. Whether or not this is normally an irreciprocal permeability, as Conklin has shown it to be in the frog, remained to be demonstrated.

This series of experiments, designed to test the permeability of the capillaries to extravascular protein, was carried out in the course of other experiments cited above. The thoracic duct was cannulated and all lymphatics entering the venous confluence on the left side were ligatured close to their entrance. The left cervical lymphatic vessel was then cannulated higher up in the neck. All lymphatic entrances on the right side were likewise tied and the right cervical lymphatic vessel was cannulated. By means of these operations, lymph was prevented from returning to the circulation and the presence of injected protein in the blood could only mean that it had passed back through the capillary endothelium. Blood samples were taken from the femoral artery, and the serum was tested with anti-horse rabbit serum for the presence of horse serum.

In this group there were four experiments in which the horse serum was injected subcutaneously. The results are tabulated in table 4. No absorption of serum in periods up to 7 hours was found. From this group of experiments we can but conclude that the capillaries of the subcutaneous tissues are permeable to protein in one direction only, and that protein once having passed from the blood through the capillary endothelium cannot pass back but must return to the blood stream by way of the lymphatic system.

These results are contrary to those of Bulloch (1898). It had been our expectation that a slight amount of direct absorption of horse serum by blood vessels would occur but such was never the case. It may be pointed out that Bulloch injected 100 cc. of antitoxin under the skin of the neck of an ass and that the lymphatics were not blocked. Lymph flow in the cervical region in the horse is very rapid and in spite of the fact that antitoxin was found in blood within 30 minutes, absorption may have been wholly lymphatic and not by the blood vessels.

TABLE 4

NUMBER OF EXPERIMENT	TIME OF INFILTRATION OF SERUM	AMOUNT OF INJECTION	DURATION OF EXPERIMENT	PRESENCE OF PROTEIN IN BLOOD		
				Time	Time	Time
		cc.	*hours*			
19	1:00 p.m.	18.0	7	1:00 –	3:00 –	8:00 –
22	2:15 p.m.	16.0	1	2:15 –	3:15 –	
23	12:35 p.m.	15.0	4	12:35 –	2:35 –	4:35 –
24	11:55 a.m.	15.0	4	11:55 –	1:55 –	5:00 –

5. *Passage of colloids from the peritoneal cavity to the blood capillaries.* Two experiments were done to determine whether or not serum injected into the peritoneal cavity could pass directly into the capillaries of this region. The same experimental procedure was followed in these experiments as in the previous one, i.e., all lymphatic entrances into the blood were ligatured. Horse serum (15.0 cc.) was injected into the peritoneal cavity and the blood serum was tested at half-hour intervals for the presence of horse serum. In one experiment, horse serum was definitely present in the blood sample taken 2 hours after the injection. A trace was present at the end of a half-hour. The serum continued to be present in increasing amounts in the blood for $7\frac{1}{2}$ hours. In the second experiment, horse serum was found in the blood in $1\frac{1}{2}$ hours and continued to be present for $3\frac{1}{2}$ hours, the duration of the experiment.

These experiments show that the capillaries of the peritoneal cavity are capable of absorbing protein. The capillary bed in this region is considerably more extensive than that encountered in the cervical region,

and the omental vessels, through which small particles have been shown to be directly absorbed, undoubtedly play an important part in the absorption of this extravascular protein.

One other experiment was carried out using the technique described by Batchelder, Field and Drinker (1931). The omentum of an animal whose lymphatic entrances into the blood had been ligatured was withdrawn and floated in a solution of garnierite—an insoluble nickel silicate solution—for one hour. At the end of this time the omentum was tied and cut off, the animal bled to death, and the liver removed and analyzed for nickel. Approximately 0.04 mgm. of nickel was found and the only possible way in which it could have reached the liver was through the portal vein. Thus, since the capillaries of the omentum are permeable to very small particles, it is altogether possible that at least a part of the foreign serum found in the blood after an intraperitoneal injection entered in this direct way.

Discussion. The experiments cited show a generalized though graded permeability to protein on the part of the blood capillaries in different parts of the body. Starling (1894), who recognized a much greater permeability on the part of the liver and intestinal capillaries, considered the capillaries in other regions to be practically impermeable to protein and based his theory of lymph formation on this assumption. The permeability of the capillaries to protein, which has been clearly demonstrated by Churchill, Nakazawa and Drinker (1927), by Conklin (1930), and by this investigation, modifies but does not invalidate Starling's original hypothesis. The effective colloidal osmotic pressure of the blood, i.e., the difference between the osmotic pressure of the intra and extravascular fluids is the important thing, and the variations between the capillary pressure and the effective osmotic pressure must determine filtration and absorption. The relationships between the osmotic pressure of the blood and lymph will be the subject of a future paper from this laboratory.

Considering the rapidity and ease with which protein passes from the tissue spaces through the lymphatic endothelium, the unavoidable conclusion seems to be that there is no appreciable difference between the fluid normally found in the tissue spaces and lymph itself. The fluid leaving the blood capillaries in the great subcutaneous region of the body undoubtedly contains the blood proteins. The actual transudate probably contains the blood proteins in higher dilution than does the tissue fluid, which is subject to concentration by withdrawal of water into the blood capillaries and to dilution by rapid filtration of water. Lymph, in our opinion, is tissue fluid. That taken from a cervical lymphatic represents the average tissue fluid composition of the region drained by a large group of lymphatics. In another paper (Drinker and Field, 1931) it is shown that lymph from large subcutaneous trunks in different parts of the body may vary in protein content, and, similarly, the microscopic lymphatics

in the drainage area of the neck may contribute tissue fluids of different protein concentration. Each will take the proteinized tissue fluid available at the moment. One cannot watch the rapid entrance of serum, milk, graphite particles and erythrocytes into the lymph without feeling that lymphatics promptly contain all material from the tissue spaces which is either unabsorbable or slightly absorbable by the blood vessels.

SUMMARY

1. The capillaries of the subcutaneous tissues permit the passage of protein through their endothelial walls. This protein can be detected in the cervical lymph from 1 to 2 hours after the injection.

2. Normal serum, injected subcutaneously, is rapidly absorbed by the lymphatics. This is shown by a concentration of the lymph and by the presence of foreign serum which can be detected serologically. The absorption is not due to puncture of the lymphatic vessels at the time of the injection.

3. Fat is absorbed by the subcutaneous lymphatics but much more slowly than protein.

4. The lymphatics of the peritoneal cavity which enter the thoracic duct take up protein even more readily than do those of the subcutaneous tissues.

5. The blood capillaries of the subcutaneous tissues are not normally concerned in the absorption of protein.

6. The blood capillaries of the peritoneal cavity are capable of absorbing extravascular protein. The omental vessels are probably most concerned in this absorption.

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THE CELL CONTENT OF DOG LYMPH

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The literature contains a number of normal cell counts on thoracic duct lymph, with values ranging from 1,000 to 50,000 cells per cubic millimeter. Comparatively few cell counts (table 1) have been reported on lymph from vessels draining limited areas of the body. None of the observers quoted noted red blood cells in the lymph. Because of the scarcity and irregularity of data on the cells of lymph from different parts of the body, the present observations on the leg and neck lymph of dogs are recorded.

Emminghaus (1873) has shown that leg lymph appears red when the vein from the leg is occluded. It was suggested that by further experiments of this sort information might be gained concerning the permeability of the lymph vessels to red blood cells. This, as well as other factors which may change the normal lymph cell content (as massage and inflammation) will be referred to in the results.

METHOD. Large dogs were used which had been fasted at least 18 hours. In order to cannulate the femoral vein for the injection of sodium barbital, a minimal amount of ether was given. Ether anesthesia lasted only about 15 minutes so that the animals were completely under sodium barbital before any determinations were made.

Lymph was obtained in various experiments from the following vessels:

1. The cervical vessels from the head, just below the medial retropharyngeal lymph gland (referred to as cervical 1).
2. The left cervical vessel as it enters the thoracic duct.
3. The right cervical vessel as it enters the junction of the subclavian and internal jugular veins (2 and 3 are grouped together and referred to as cervical 2).
4. The thoracic duct.
5. The lymph vessels in the femoral triangle between the artery and vein, below the groin and iliac nodes.
6. Lymph vessels on the front of the ankle.
7. A lymph vessel from the kidney (one experiment).

In most cases the lymph vessels were cannulated and the lymph collected from the cannula by a capillary pipette. Since lymph was sometimes

obtained from the leg by piercing the vessel and collecting lymph directly by a fine syringe or capillary pipette, the method of collection has been noted in table 2. For the lymph vessels cannulated a binocular microscope was not found to be necessary. In order to avoid sedimentation of cells, lymph which had stood for any length of time in the cannula was discarded. Massage was excluded as far as possible but for the small leg vessels and in other cases where lymph flow was sluggish gentle pressure was sometimes necessary in order to obtain enough lymph for the determinations. For differential counts, smears were stained with Wright's stain.

RESULTS. *Normal cell counts.* The results are summarized in table 2. The figures on cervical 1 lymph are based on longer series of counts than those on other kinds of lymph and are therefore probably more accurate.

TABLE 1

Summary of lymph cell counts on lymph from other vessels than the thoracic duct

OBSERVER	KIND OF LYMPH	TOTAL WHITE CELL COUNT	DIFFERENTIAL	ANIMAL USED
Davis and Carlson (1909)	Neck lymph	5,000–24,722	Practically 100 per cent small mononuclears	Dog
Winternitz (1895)	Leg lymph (thigh)	403–1173		Dog
Menkin and Freund (1929)	Lymph from efferent vessel of axial lymph node	9,000–55,000	100 per cent lymphocytes	Rabbit
Florey (1927)	Lymph from efferent vessel from mesenteric lymph gland	1,400–36,875	Mostly lymphocytes and a small number of mononuclear cells	Cat

The cervical lymph, which in some part flows through large lymph nodes, has as many if not more cells than the thoracic duct lymph. Lymph from the vessels of the groin shows comparatively few cells. The presence of any cells in these vessels may be accounted for by the fact that the lymph may have gone through a few small lymph nodes at the back of the leg. The fact that the lymph from the ankle, which has passed through no lymph nodes, contains practically no cells would indicate that normally the cells enter the lymph from the lymph nodes and not by diapedesis from the blood vessels nor from the tissue spaces.

The differential counts in table 2 represent the average of counts of several smears of lymph from each part of the body. The white cells in the lymph were practically all lymphocytes. Red blood cells appeared in cervical 1 lymph very seldom. Thoracic duct lymph, however, was usually

TABLE 2

Cell content of dog lymph

DATE	TOTAL WHITE CELL COUNT	PER CENT LARGE LYMPHOCYTES	PER CENT SMALL LYMPHOCYTES	PER CENT POLYMORPHONUCLEARS	RED BLOOD CELLS	PER CENT PROTEIN	METHOD OF COLLECTION
Cervical 1							
10/ 6/30	12,350	4	95	1	−	1.45	
10/11	8,500	1	99	0	−	1.22	
10/14	8,975	2	98	0	−	2.15	
10/16	24,150	5	95	0	−	2.67	
10/20	4,075	2	98	0	−	1.22	
10/24	27,325	4	95	1	−	1.84	
10/28	8,750	5	95	0	+	2.22	
1/ 2/31	8,050	7	93	0	−	1.20	
Average	12,772	4	95				
Cervical 2							
10/30	5,200	3	95	2	+++		
11/ 6	20,600	1	99	0	++	2.58	
11/12	68,600					3.38	
11/25	18,675	4	96	0	−	2.17	
12/ 2	7,200	0	100	0	+++	3.80	
12/ 4	2,800	1	99	0	+	1.66	
12/ 7	19,300	3	97	0	++	2.10	
Average	20,512	2	98				
Thoracic duct lymph							
10/30/30	9,100	4	95	1	++++		
11/ 6	12,250	4	96	0	+++		
11/12	4,800	2	98	0	++	4.82	
11/19	500	7	88	5	++++	4.47	
12/ 2	6,000	1	99	0	++++	3.27	
12/ 4	2,800	3	97	0	++++	3.17	
Average	5,908					3.93	
Kidney lymph							
11/25	1,200					3.79	
Leg lymph (at femoral triangle)							
10/ 6	0					1.62	By cannula
10/20	420				++	0.70	By syringe and cannula
10/24	400				−		By syringe
10/28	0				++		By pipette
11/ 6	2,500				+	1.34	By pipette
11/12	200				++		By pipette
11/19	150				+		By pipette
Average	520					1.22	By pipette
Leg lymph (at ankle)							
11/25	0				+	1.86	By cannula
12/ 7	100				+		By pipette
1/ 3/31	0				+		By cannula

bloody and cervical 2 lymph often so. Increased permeability of the vessel is also suggested by an increased protein content. From the small vessels of the groin where lymph was collected without cannulating the vessel slight blood contamination may have occurred.

Effect of venous occlusion. Since sodium barbital was given through a cannula in one of the femoral veins, it was necessary to keep this vein tied off throughout the experiment. As in Emminghaus' (1873) experiments it was noticed that lymph from the leg with the vein occluded appeared reddish as compared with the colorless lymph of the leg with the vein untied. On the leg lymph a determination of this difference by smears was rather difficult since control lymph obtained without occlusion usually contained red cells. The results seemed to be more definite when marked congestion and swelling of the leg were observed. In one such experiment lymph, which contained only a few red cells, became very bloody in less than 30 minutes after the vein was tied.

Effect of massage. Experiments on cervical lymph confirm the observation of Davis and Carlson (1909) that massage causes a marked increase in the number of cells in the lymph.

Before massage *cells per cmm.*	After massage *cells per cmm.*
6,850	24,900
11,100	25,200

In these cases the lymph glands of the neck were massaged and undoubtedly contributed the increase in lymphocytes. On the leg, where there were no glands massage did not greatly change the number of cells in the lymph.

In two experiments massage was continued at short intervals and a series of cell counts made. During the period of massage the number of cells progressively decreased, probably indicating a washing out of cells from the lymph glands.

	Expt. 16	*Expt. 18*
Before massage	2,900	9,400
After massage	12,800	7,800
After massage	12,700	6,400
After massage	6,700	5,700
After massage	5,200	
After massage	4,700	
After massage	3,100	

In one case the lymph contained slightly more polymorphonuclear leucocytes after prolonged massage.

Effect of injection of graphite. In one experiment a suspension of graphite was infiltrated under the skin of the hind foot. Lymph collected at the ankle 2 to 4 hours after injection showed a majority of polymorphonuclear

leucocytes and many red blood cells. The results of this experiment suggest a condition similar to that found in inflammation by Winternitz (1895) and Menkin and Freund (1929).

SUMMARY

1. The numbers of cells in the lymph from different parts of the body as shown by these experiments agree with the data of other workers and show irregularities similar to theirs. The difference in cell content of lymph passing through areas with and without lymph nodes is clearly demonstrated.

2. The cervical lymph which had passed at least in part through lymph glands was found to contain 2,800 to 68,600 white cells per cubic millimeter whereas leg lymph which had passed through few if any nodes contained 0 to 2,500 cells.

3. By a short period of massage of the cervical lymph glands the white cell count of the cervical lymph could be greatly increased and gradually fell on continued massage.

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INFLUENCE OF THE SYMPATHETICS ON MUSCLE GLYCOGEN

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In recent years a large volume of work has appeared on the relation of the sympathetics to muscular contraction. Following the suggestions of Hunter and Royle (Royle, 1924), numerous attempts were made to relate these nerves directly to skeletal muscle tonus. Although the anatomical mechanisms seemed sufficient few recent workers have been able to detect either an immediate loss of tonus after sympathectomy or any modification of plastic tonus after decerebration of such animals. The papers of Kanavel, Pollock and Davis (1925), Meek and Crawford (1925), Forbes and others (1926) and Tower (1926) may be consulted in this connection.

While the work on tonus was proving negative, Orbeli was, however, succeeding in showing that the sympathetics were able to modify striated muscular contraction and others were studying the effect of these nerves on the metabolism of cold blooded muscle.

Orbeli's experiments (1923) demonstrated clearly that sympathetic stimulation may either delay the onset of fatigue in a frog's gastrocnemius, or aid in its recovery, and that the sympathetic effect at the end of the motor nerve is independent of any oxidative recovery process. Similar results were obtained by Maibach (1928) and Labhart (1929) on frog muscle, their results being especially significant since the sympathetic effects appeared only after a latent period, persisted after cessation of stimulation and were unaccompanied by any visible motor response.

The extension of such studies to mammalian muscle has been made by Coates and Tiegs (1928) and Baetjer (1930). The former found with dogs and goats that the tibialis anterior and gastrocnemius fatigued much more rapidly when deprived of their sympathetic supply than did the controls. However, their animals had been sympathectomized for three to four months and there may have been some dystrophic changes in the muscle. Baetjer stimulated the tibialis anterior in cats with a series of single induction shocks applied to the anterior roots. Simultaneous stimulation of the abdominal sympathetic chain caused either an increase or a decrease in height of contraction. The decrease she found to be due to vasoconstriction. The increase she concluded was not due to an increased blood supply nor to any substance carried by the blood but rather to stimulation

of nerve fibers in the chain other than vasomotors, which affected the muscle in some unknown way, producing heightened contractions.

One of the earliest to search for a possible metabolic influence of the sympathetic on muscle was Ernst (1915) who stimulated the sciatic plexus of curarized frogs for 30 to 60 minutes and found an hour later that in five out of seven cases there was a decrease of 2 to 16 per cent glycogen on the stimulated side.

Büttner (1926) compared the glycogen content of frog gastrocnemii 18 to 60 hours after unilateral section of the sympathetic rami. In all of his six experiments he found the operated side to have 11 to 62 per cent more glycogen. Hoffman and Wertheimer (1927) unilaterally sectioned the

TABLE 1

Muscle glycogen of normal rabbits

RABBIT NUMBER	MUSCLE	GLYCOGEN PER 100 GRAMS OF MUSCLE		DIFFERENCE
		Right	Left	
		mgm.	*mgm.*	*per cent*
26	Hamstrings	276	268	2.9
	Quadriceps	288	310	7.0
34	Hamstrings	279	290	3.9
	Quadriceps	347	367	5.4
37	Hamstrings	321	296	7.7
	Quadriceps	293	302	2.9
38	Hamstrings	343	371	7.5
	Quadriceps	285	302	5.6

sympathetic rami to the hind legs of frogs and later killed them with strychnine. The glycogen content of the gastrocnemii and vasti was decidedly greater on the operated side. When the operated frogs were subjected to a temperature of 37° for 2.5 to 48 hours instead of the strychnine, the same kind of result was secured. When the rami were unilaterally cut in summer frogs which were still kept at 37°, there was a significant increase in glycogen on the operated side. Hoffman and Wertheimer concluded that the heat and strychnine had stimulated the sympathetic nervous system, the activity of which had caused the diminution of glycogen on the unoperated side.

In our study of the metabolic influence of the sympathetic nervous system on skeletal muscle we have removed the lumbar chain aseptically

on one side in rabbits and dogs, and after a recovery period, have determined the glycogen content of muscles from the two hind legs.

METHOD. The animals were killed by a blow on the occiput and the muscles removed usually in two groups; the quadriceps femoris and the hamstrings (the biceps femoris, semi-tendinosus and semi-membranosus).

The muscles were weighed, cut into a few pieces and dropped into boiling 60 per cent potash. In less than 15 minutes after the death blow the muscles were in the hot alkali. After digesting for three hours the samples were made up to volume (100 cc.) and aliquots (10 cc.) taken for analysis. The aliquot was placed in a 50 cc. centrifuge tube, 30 cc. of alcohol added, the glycogen allowed to precipitate by standing in a refrigerator room. The tube was then centrifuged, part of the supernantant fluid siphoned off and the rest filtered. The precipitate was then taken up in water and reprecipitated as before. The glycogen precipitate was hydrolized with 1 per cent HCl by standing in a boiling water bath for 4 to 5 hours. The reducing sugar was determined by the Folin modification of the Folin and Wu colorimetric method. All analyses were made in duplicate and in questionable cases in triplicate. To reduce the inevitable errors of the method in making the comparison of the glycogen content of the two sides the procedures were carried out on the homologous muscles of the two sides simultaneously.

Glycogen determinations in normal muscle. In table 1 are presented analyses from homologous muscles of opposite sides. It will be noted that the percentage difference for the two sides varies from 2.9 to 7.7 per cent. From these data as well as reports from other workers we feel that any difference of glycogen content greater than 8.0 per cent is quite likely a significant one.

The effect of sympathectomy on the glycogen content of muscle. Since the results of electrical stimulation of the sympathetics are apt to be confused by the anesthesia and vasomotor reactions, we have approached the problem by removing aseptically the lumbar sympathetics on one side in rabbits and dogs. In some cases the removal included the last thoracic and first sacral ganglia. The animals were allowed 28 to 86 days in which to recover, at which time they were killed by a blow on the occiput. The blood vessels in the neck were then cut in order to bleed the animal. Although in some cases the muscles of the operated side were removed first and in others the controls, the order seemed to make no difference in the results.

The glycogen content of muscles from the operated and control sides is given in table 2.

These experiments show with one exception (rabbit 1) that muscles

TABLE 2

Comparison of glycogen content of muscles in unilaterally sympathectomized rabbits and dogs

RABBIT NUMBER	RECOVERY PERIOD	MUSCLE	GLYCOGEN PER 100 GRAMS OF MUSCLE		DECREASE ON OPERATED SIDE
			Operated	Control	
	days		*mgm.*	*mgm.*	*per cent*
1	30	Hamstrings	282	242	+16.6
		Quadriceps	235	243	3.2
6	36	Hamstrings	232	342	32.1
		Quadriceps	325	323	
7	21	Hamstrings	135	142	4.9
		Quadriceps	106	130	18.4
8	86	Hamstrings	325	371	12.4
		Quadriceps	476	483	1.4
		Adductors	365	399	8.5
9	65	Hamstrings	192	240	20.0
		Quadriceps	340	345	1.4
		Adductors	300	313	4.1
14	46	Hamstrings	098	136	27.9
		Quadriceps	175	186	5.9
		Adductors	111	117	5.1
DOG NUMBER					
22	20	Semi-membranosus, semi-tendinosus	704	749	6.0
		Biceps femoris	715	716	
		Quadriceps	595	678	12.2
24	26	Biceps femoris, semi-tendinosus	499	516	3.2
		Semi-membranosus	331	349	5.1
		Quadriceps	439	501	12.3
25	24	Semi-tendinosus, semi-membranosus	542	562	3.5
		Biceps femoris	428	487	12.1
		Quadriceps	431	435	
27	48	Hamstrings	370	429	13.7
		Quadriceps	345	429	19.5

deprived of their sympathetic nerve supply contain less glycogen than their normal homologues of the opposite side. This fact is in confirmation of Britton's report (1930) that sympathectomy causes a loss of muscle glycogen. Büttner, however, had found that section of the sympathetic rami in frogs resulted in more glycogen on the operated side. However, one must remember that the time element was only 18 to 60 hours in his experiments. We would suggest that in these short periods of sympathectomy, the improvement in nutrition following the vascular changes might hide other factors. Also, Beattie, Beattie and Milroy (1930) concluded from their sympathectomy experiments on dogs that the glycogen content of the sartorius and biceps femoris was only slightly affected by such procedure. However, the magnitude of the glycogen difference between the normal and the sympathectomized biceps femoris reported by them is about the same as reported in table 2.

TABLE 3

Effect of hyperthyroidism on trophic influence of sympathetics in rabbits

RABBIT NUMBER	RECOVERY PERIOD	MUSCLE	GLYCOGEN PER 100 GRAMS MUSCLE		DECREASE ON OPERATED SIDE
			Operated	Control	
	days		*mgm.*	*mgm.*	*per cent*
2	48	Hamstrings	125	170	26 4
		Quadriceps	186	184	
18	38	Hamstrings	345	457	24 5
		Quadriceps	396	465	14 8
19	13	Hamstrings	568	731	22 3
		Quadriceps	793	908	12 6

Relation of hyperthyroidism to sympathetic action. In view of the results obtained by Hoet and Marks (1926) on the depletion of muscle glycogen by feeding thyroid we tried similar experiments to see if this procedure would accentuate the effect of sympathectomy. In one case, no. 2 of table 3, 16 grains of U. S. P. thyroid were fed daily for 2 weeks to a unilaterally sympathectomized rabbit.

The glycogen content of all the muscles examined was markedly reduced, the reduction in the hamstrings on the operated side being considerably greater than in the quadriceps. In two other animals, rabbits number 18 and 19, the two weeks of thyroid feeding preceded the operation. Undoubtedly the muscles had been well depleted of glycogen at the time of operation, and the results show that during the recovery period both sets

of muscles show glycogenesis. The interesting point is, however, that the operated side is handicapped in this regeneration, the hamstrings being more affected than the quadriceps. These thyroid feeding experiments have emphasized the differences in glycogen content between normal and sympathectomized muscle, which were already apparent in the data of table 2.

In our experiments the animals were allowed from 4 to 6 weeks in which to recover, by which time it was thought that all vasomotor disturbances had been corrected. Boshamer (1925) reported that he could find no vaso-dilatation in frogs five days after section of preganglionic fibers, and when the postganglionic fibers were sectioned the vaso-dilatation only lasted about twenty-four hours. Tower found that the temperature of the foot-pads of dogs was the same by the second week after unilateral removal of stellate ganglion. McCullagh and co-workers (1930) found that at the end of two weeks after unilateral lumbar sympathectomy in dogs the cutaneous digital temperatures of the two hind legs were the same. Britton measured the rate of blood flow from the extremities twenty-four to sixty-two days after unilateral removal of the stellate ganglion and the lumbar chain. His conclusions were that the muscle glycogen changes did not appear to depend alone if at all on vascular shifts.

In our animals there was always a very marked difference in temperature of the two thighs immediately after the operation. Usually by the end of ten days no difference was felt. In two cases the temperature of the sympathectomized muscle was compared with the normal by means of thermocouples. Under local anesthesia these were inserted directly into the muscle. Eight readings at intervals of five minutes were made on both hind legs simultaneously. In a rabbit thirty-six days after unilateral removal of the lumbar chain the semi-membranosus of the operated side was warmer by 0.38°C. The day following it was cooler by 0.3°C. In a dog twelve days after a similar operation the quadriceps was 0.31°C. higher on the operated side. These variations were all within the normal range. These observations as well as those cited in the literature indicate that the vasomotor changes induced by sympathectomy had been corrected for a considerable period before our animals were sacrificed for glycogen analysis.

Although after some weeks there are not sufficient temperature changes present to account for a decreased glycogen content in sympathectomized muscle, it cannot be denied that for a few days after operation considerable variations of temperature existed. To test the effects of these early dilatations we have studied two rabbits two and six days after operation. We have also induced a greatly increased circulation in the muscles of one side

in two rabbits by means of diathermy. The fur was removed and the electrodes held firmly in place with rubber bands. The current was turned on for twenty minutes, alternating with twenty minutes' rest, for forty hours in the first case and for fifteen hours in the second.

The results of these experiments were not entirely consistent. The animals sympathectomized for two and six days both showed a loss of glycogen in the denervated hamstrings, averaging about 10 per cent. The temporary increases in blood supply did not then prevent the usual decrease in these muscles. In one rabbit subjected to diathermy for forty hours, there was an increase of glycogen in all the muscles concerned while in the rabbit subjected to fifteen hours diathermy, there was no difference from the control side. Since increased blood supply did not prevent a lowering of the glycogen content in sympathectomized muscle nor cause it in intact muscle, it is very doubtful if it had any definite effect.

This leaves us with the generalization that sympathetic denervation causes a lower muscle glycogen content not because of vasomotor changes but because of the deprivation of its direct glycogenic influence.

General discussion. The results obtained with the animals from which one of the sympathetic chains has been removed, show undoubtedly that the denervation causes a diminution in the glycogen content of the muscles involved. The diminution involves certain muscle groups to a greater degree than others. It is most marked in the hamstrings of the rabbit and the quadriceps of the dog. These muscle groups represent the tonic muscles in the respective animal species. This glycogenic function of the sympathetic nervous system represents a superimposed form of control because muscles do accumulate glycogen when deprived of its influence but such muscles are handicapped in the restoration or maintenance of their glycogen and the postural muscles are affected to the greatest extent. There is ample proof that muscle tonus is not directly maintained by the sympathetic nervous system. However, muscle tonus may be supported indirectly by the glycogenic activity of the sympathetic nerve fibers, in aiding the postural muscles to maintain their supply of available energy. The sympathetics may thus have a relation to skeletal muscle tonus, although quite different from that originally proposed.

From the results of this study it would seem to follow that sympathetic impulses cause deposition of glycogen in muscles. This function of the sympathetics might explain the results obtained by Orbeli, Baetjer and others in augmenting the height of contraction or hastening recovery from fatigue in an active muscle. One might say that the electrical stimulation of the sympathetic nerves, in their experiments, had either produced or made available an increased supply of glycogen to the contracting muscle.

Certain observations opposed to this point of view must, however, not be overlooked. Wertheimer, Cori and others have found that adrenalin causes a disappearance of muscle glycogen and Wertheimer has stated that this does not occur in sympathectomized muscle. Furthermore we have ourselves tried to ascertain the effect of electrical stimulation of the lumbar chain on muscle glycogen. Our results are too variable to be conclusive but they do seem to indicate a loss of glycogen on stimulation. It would seem, then, that normally impulses passing down the sympathetics produced a glycogenetic effect while those produced artificially were glycogenolytic. Such results, apparently contradictory, could be explained on the assumption of two sets of fibers in the nerves.

SUMMARY

1. Unilateral removal of the lumbar sympathetic chain in dogs and rabbits results in a lower glycogen content of the muscles on the operated side.

2. The reduction of muscle glycogen following unilateral sympathectomy is greater in rabbits that are in a state of hyperthyroidism. Such muscles may replenish their glycogen after the hyperthyroidism subsides but not to the extent that the normally innervated muscle does.

3. Glycogenesis in the tonic muscles seems to be aided directly by the auxiliary glycogenic function of the trophic fibers in the sympathetic nerves.

4. Increased blood flow to a limb does not of itself seem to cause any decrease in muscle glycogen. The diminution in muscle glycogen following sympathectomy is due only to an interruption of the glycogenic influence of the sympathetics.

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THE SECRETION PRESSURE OF THE AGLOMERULAR KIDNEY

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In all investigations on glomerular kidneys, the secretory pressure of the kidney has been found to be below that of the arterial blood pressure (Cushny, 1926). As has been frequently pointed out, this is very different from the salivary gland in which the duct pressure may exceed by a considerable margin the arterial blood pressure. In the present investigation it will be shown that the secretion pressure of the aglomerular kidney resembles the behavior of the salivary gland, more than that of the glomerular kidney, in that it may rise above the arterial blood pressure.

The fact that the pressure in the salivary duct would rise above the arterial blood pressure was first discovered by Ludwig in 1851. In one of his typical experiments on a dog, the carotid blood pressure was 108.5 mm. Hg, while the secretion pressure of the gland was 190.7 to 196.5 mm. Hg.

It is also to be noted that the pancreas and liver behave in a similar, though not completely identical, manner. The pancreas (Henry and Wollheim, 1877; Herring and Simpson, 1909) will secrete to a pressure several times higher than the pressure in the veins draining the gland. In another series of experiments of Pavlov (1888), in which the blood pressure was lowered by bleeding, the secretory pressure of the pancreas rose above the general blood pressure; for example, when the blood pressure had dropped to 90 mm., the secretion pressure of the gland rose from 89 to 96 mm. The liver (Friedlander and Barisch, 1860; Heidenhain, 1883; Herring and Simpson, 1907) will also secrete bile at a pressure several times higher than the blood pressure in the portal vein.

Essentially there is perhaps no great difference in the behavior of the secretory pressures of these glands. In all three, secretion can be poured out against pressures considerably higher than that occurring in the capillaries in these glands.

For the experiments herein reported, the toad fish (*Opsanus tau*) has been used. It was first demonstrated by Marshall (1929) that the kidney of this fish is aglomerular. It has also been shown by Marshall (1930) that secretion occurs in this kidney. One would, therefore, expect that this kidney might behave more or less as a true secretory gland.

The method of study has been that devised for a study of diuretic drugs which will be reported later. In most of the experiments the fish were anesthetized lightly with urethane. In a few no anesthetic except 1 to 2 cc. of 0.1 per cent procaine hydrochloride was used for opening the abdominal cavity. The fish were placed ventral side up in a box containing aerated sea water. The arrangement allowed for unobstructed respiration which occurred throughout the time of experimentation. Both ureters were isolated, and cannulated. The large coeliac artery was also isolated, and it was cannulated about 1 cm. from the point of its origin from the dorsal aorta and the posterior pair of efferent branchial arteries. This would indicate that the pressure was practically identical with that of the dorsal aorta.

Water manometers were used to measure the pressures. In the case of the artery, saline containing heparin was used. Two long glass tubes of 2 mm. bore were attached, one to the cannula in the artery, and one to one of the ureteral cannulae. Millimeter scales were placed alongside of these tubes measuring the distance from the level of the heart. The pressure against which the aglomerular kidney could secrete was then measured by varying the level in the ureteral manometer and waiting a sufficient time to determine the flow of urine. The pressures described in this paper are expressed in millimeters of mercury. In some of the experiments a solution of magnesium sulphate was injected intravenously to produce a more rapid flow of urine.

A typical protocol of an experiment is given below:

Toad fish ♂. Weight 540 grams. December 29, 1930. 10:00 a.m. Fish placed in urethane solution for 15 minutes. 10:15 to 11:00 a.m. Cannulae inserted, and blood pressure system opened. 11:00 to 12:00 noon. Blood pressure rose from 10.3 to 12.3 mm. Hg. During this time interval the period of "reflex anuria" disappeared. No diuretic drug was injected to increase urine flow. Respiration 10 to 14 per minute throughout experiment.

LEFT KIDNEY SECRETING AGAINST PRESSURE		ARTERIAL BLOOD PRESSURE	RIGHT KIDNEY SECRETING NORMALLY (NO PRESSURE) FLOW OF URINE
Flow of urine	Pressure in ureteral cannula		
	mm. Hg	*mm. Hg*	
9 cu. mm. in 18 minutes	7.9	12.8	7.5 cu. mm. in 15 minutes
12 cu. mm. in 17 minutes	13.5	13	15 cu. mm. in 20 minutes
6 cu. mm. in 14 minutes	16.2	13.5	7.5 cu. mm. in 12.5 minutes
12 cu. mm. in 22 minutes	17.6	13.5	15 cu. mm. in 28 minutes
12 cu. mm. reverse flow in 14 minutes	19.5	13.5	15 cu. mm. in 27 minutes
18 cu. mm. in 39 minutes	14.2	13.5	15 cu. mm. in 27 minutes

The following table summarizes the findings in seven fish.

	FISH 1	FISH 2	FISH 3	FISH 4	FISH 5	FISH 6	FISH 7
Arterial blood pressure, mm. Hg....	11.1	11.3	13.5	17.3	15.0	16.1	14.4
Maximum ureteral pressure showing secretion of urine, mm. Hg...	16.0	15.6	17.6	19.0	18.1	18.1	17.0

As can be seen from the experiments described above, the secretion pressure of the aglomerular kidney rises above the dorsal aortic blood pressure. Considering the fact that the main blood supply to this kidney is venous the pressure in the kidney capillaries is much lower, probably only one-fourth of the dorsal aortic pressure. This means that the secretion pressure can be four to five times as high as the blood pressure in the kidney capillaries. Even when the pressure in the ureter is raised, the rate of urine flow is comparable to that in the opposite kidney secreting against no pressure.

SUMMARY

It has been shown that in the aglomerular kidney (toad fish), the secretion pressure taken in the ureter can be greater than the dorsal aortic blood pressure.

I desire to express my grateful appreciation to Dr. E. K. Marshall, Jr., for suggesting this problem, and for his interest and helpful criticism shown throughout the work

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HISTAMINE AND PILOCARPIN IN RELATION TO THE GASTRIC SECRETION[1]

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The composition of the gastric juice and its variations in response to different stimuli have been well known for many years, but the intricate mechanism controlling these variations was not clearly understood. In the past few years, however, certain data have been obtained which tend to a more comprehensive view of the subject. Thus there are certain indications that the qualitative changes in the composition of the gastric juice secreted under different conditions are due to unequal quantitative activity of the different groups of secretory elements in the gastric glands (Babkin, 1929, 1930a). The same principle is illustrated in the experiments recently performed by one of us (A.M.V., 1931), in which it was demonstrated that different strengths of electrical stimulation of the vagus nerve produced gastric juice of varied composition. Thus weak stimulation activated a secretion rich in mucus, low in acidity and having a moderate digestive power. Strong stimulation on the other hand resulted in a copious gastric secretion poor in mucus content with high acidity and high digestive power.

The present work is based on the same principle as the previous investigation, and demonstrates experimentally the possibility of stimulating independently or simultaneously the different cytological elements of the gastric glands. This has been accomplished by the use of the two chemicals, histamine and pilocarpin.

METHODS. Heidenhain and Pavlov pouch dogs were employed, as well as a dog with esophagotomy and gastric fistula. The free and total HCl were determined by the titration method, the peptic power by Hawk's modification of Metts' method. The chlorine concentration was determined by the method of Wilson and Bald. The volume of visible mucus was estimated by centrifuging the juice. Histamine and pilocarpin were injected subcutaneously, histamine in doses ranging from 0.75 to 1 mgm. and pilocarpin in doses of from 4 to 7 mgm.

[1] The preliminary report of this work was published by B. P. B. in the Canadian Medical Association Journ., 1930, August, xxii, 268–272.

EXPERIMENTAL RESULTS. *Histamine.* Following the injection of histamine gastric secretion was activated, the juice obtained having a high acidity and a diminishing enzyme concentration. A second dose of histamine increased the volume of the secretion and yet the enzyme content continued to decrease (see table 1).

TABLE 1

Repeated injections of histamine

Dog with esophagotomy and gastric fistula. Weight 20 kilo; 1 mgm. histamine injected subcutaneously; 30 minutes later dose repeated.

SAMPLE	TOTAL TIME	VOLUME	FREE HCl	TOTAL HCl	Cl	PEPTIC POWER	REMARKS
	minutes	*cc.*	*gram per cent*	*gram per cent*	*mgm. per cent*	*Metts' units*	
1	60	Mucus					At end of this period reaction in stomach weakly alkaline
							Histamine, 1 mgm.
2	5	0.8					Thick yellow mucus expelled from tube
3	5	8.0			606.0	384.1	Bile admixed
4	5	6.0	27.7	32.1	602.0	153.7	Clear juice
5	5	20.5	40.8	45.2	602.0	64.0	
6	5	24.0	45.9	50.1	615.4	19.3	
7	5	23.0	48.0	51.1	602.0	19.3	
							Histamine, 1 mgm.
8	5	8.5	48.9	52.5	612.4	16.0	
9	5	19.0	48.9	52.2	612.4	16.0	
10	5	24.0	50.3	53.2	609.6	10.2	
11	5	21.0	51.1	51.4	609.6	10.2	
12	5	17.0	48.9	51.2	609.6	4.0	
13	5	15.5	51.2	53.5	602.0	Trace	
14	15	43.0	48.9	51.3	601.2	Trace	
15	15	26.5	45.2	48.9	581.2	Trace	
16	15	12.4	40.8	43.8	575.6	Trace	
17	15	4.8	35.0	39.0	564.2	1.2	
18	30	6.0	31.4	38.6	439.4	4.0	

From the above it would seem that the enzymes obtained following histamine stimulation were simply washed out from the glands. The process seems to be a passive one. It is quite different from the active secretion of organic substances and enzymes under the influence of other stimuli. This effect of histamine appears to be independent of the parasympathetic nervous system, as is suggested by some experiments on atropinized dogs. In one experiment 1 mgm. histamine, injected into an atropinised (2 mgm.) dog, with a Heidenhain pouch and gastric fistula, produced 7.4 cc. of gas-

tric juice over a period of 85 minutes. In another analogous experiment 1 mgm. histamine produced 8.85 cc. over a period of 75 minutes. In a control experiment, using histamine alone, 8.8 cc. were obtained in 80 minutes. Thus the volume of the secretion following histamine administration is but slightly affected by atropinisation. The enzymes showed the usual diminishing curve. The results with respect to atropin are in accordance with the findings of previous investigators (see Feldberg and Schilf, 1930).

TABLE 2

Histamine and pilocarpin

Dog with esophagotomy and gastric fistula. Weight 20 kilo. During the 20 minutes' collection period prior to injection of histamine, 0.5 cc. of very weakly acid mucus was secreted; 6 mgm. of pilocarpin were injected 30 minutes after injection of 1 mgm. of histamine.

SAMPLE	TOTAL TIME	VOLUME	FREE HCl	TOTAL HCl	PEPTIC POWER	FERMENT UNITS	REMARKS
	minutes	*cc.*	*gram per cent*	*gram per cent*	*Metts' units*		
1	15	26.0					*1 mgm. histamine* Bile sample contaminated
2	5	22.0	0.39	0.42	36.0	792.0	Trace bile
3	5	17.5	0.43	0.47	16.0	280.0	Clear
4	5	15.0	0.44	0.49	4.0	60.0	Clear
							6 mgm. pilocarpin
5	5	11.5	0.46	0.48	16.0	240.0	
6	5	10.5	0.43	0.46	23.0	241.5	Salivation started
7	5	10.0	0.42	0.45	231.0	2310.0	Much salivation
8	5	9.8	0.42	0.45	196.0	1920.0	
9	5	10.5	0.42	0.47	196.0	2058.0	
10	5	9.5	0.45	0.47	163.7	1555.1	
11	5	11.0	0.44	0.47	92.1(?)	1013.1	
12	15	9.5	0.20	0.26	163.8	1556.1	Salivation less
13	15	3.5	0.36	0.40	364.0	1274.0	
14	15	1.1	0.36	0.39	272.0	299.2	Slight salivation

Pilocarpin. Quite different results were obtained if during the histamine secretion pilocarpin was injected. The influence of the pilocarpin was manifested in a certain increase in the volume of the secretion or its prolongation, a slightly lowered free and total acidity and a tremendous augmentation of the peptic power. (See table 2.)

In table 2 the peptic power for a given period of time is expressed in ferment units (calculated by multiplying Metts' units by the total volume). It illustrates the enormous increase in the peptic power following the introduction of a new stimulus and indicates that there was an active output of enzymes. Similar results were obtained when Pavlov and Heidenhain pouch dogs were used (Babkin, 1930b). The chlorine concentration,

which quickly reached its normal value in these experiments during histamine secretion, did not undergo any marked change after the injection of pilocarpin.

During the course of this investigation it was observed that when pilocarpin alone was administered to a dog, there would be profuse salivation, regurgitation of duodenal juices into the stomach, sometimes vomiting, extreme restlessness and frequently defecation. If however histamine had been given previously, the salivation seemed to be less profuse, the dog remained quiet and there was no vomiting or defecation.

To ascertain the accuracy of this observation experiments were performed on salivary fistulae dogs in our laboratory by Dr. H. Baxter, whom we have to thank for the following data:

The salivary secretion over 70 minutes after 7 mgm. pilocarpin was 60.3 cc.

The salivary secretion over 45 minutes after 1 mgm. histamine was 3.2 cc.

The salivary secretion over 70 minutes after 1 mgm. histamine and 7 mgm. pilocarpin was 40.2 cc.

Thus it would seem that histamine diminishes to a certain extent the activity of pilocarpin on the salivary glands. These data are in agreement with the results obtained by Miss MacKay (1927) on anesthetised dogs and cats. She found that the injection of histamine following pilocarpin inhibited the secretion of the submaxillary saliva and the flow of blood through the gland.

Discussion. The experimental data reported above afford evidence that histamine stimulates selectively those cytological elements of the gastric glands which secrete water, HCl and some other inorganic constituents of the juice. The enzymes which are produced simultaneously by the gastric glands do not seem to be actively secreted under histamine stimulation. The enzymes present in gastric juice of histamine activation are probably "washed out" from the lumen of the glands or from the cells. However, the precise mechanism of this phenomenon remains unknown. Independently from us, Gilman and Cowgill (1930) came to the same conclusion with regard to the action of histamine. As was already known (Tschurilow, 1894; Zitowitsch, 1902), pilocarpin chiefly activates the production of organic substances and enzymes in the gastric juice. In addition it initiates a secretion of the liquid parts and HCl though to a relatively less extent. Hence pilocarpin, acting through the medium of the parasympathetic nervous system, produces mainly a so-called *trophic effect* (Heidenhain).

Histamine is used daily by clinicians as a gastric test meal. As is apparent from the above data, it stimulates chiefly the parietal cells producing HCl and to a much lesser degree (if at all) the peptic and mucoid cells. Therefore the histamine gastric test meal, when employed as a diagnostic

test of gastric function yields information concerning the state of only one group of the cytological elements comprising the gastric mucosa. Hence it is inadequate (Babkin, 1930b).

It might be more desirable to employ a combined histamine-pilocarpin test for gastric function in humans, for the following reasons:

1. The combination of the two drugs activates (in dogs) all the known cytological elements of the gastric mucosa in a manner which approximates the gastric secretion obtained under normal stimuli.

2. There are certain indications that the clinical study of peptic function may be of greater diagnostic value than that of HCl. Polland and Bloomfield (1930), using the histamine test meal studied the HCl and peptic power of the gastric juice in normal individuals and in patients suffering from various gastric and general diseases. They concluded that "it seems that the low pepsin output is a more delicate index of gastric damage than low acid values."

3. The objectionable features of pilocarpin (i.e. profuse salivation and hypermotility of the intestines) are not so prominent when its administration (in dogs) follows the use of histamine.[2]

CONCLUSIONS

1. Histamine activates selectively those cytological elements of the gastric glands which secrete water, HCl and other inorganic constituents, without affecting enzyme secretion.

2. Pilocarpin activates chiefly the production of the organic substances and enzymes of the gastric juice.

3. Combination of histamine and pilocarpin produces a synthetic gastric juice approaching normal.

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[2] One of us, A. M. V., proposes to apply the combined histamine and pilocarpin test meal to humans in the hope of improving present diagnostic methods in stomach diseases.

FURTHER STUDIES OF THE CHEMICAL REACTIONS UNDERLYING THE COAGULATION OF THE BLOOD. THE ACTIVITY OF CEPHALIN[1]

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Our studies of some of the phenomena of the coagulation of the blood have revealed hitherto unrecognized significant reactions between lipoid, calcium and protein.

Experiments with cytozyme have confirmed the observations of Bordet in so far as they relate to the activity of cytozyme, but not his conclusions as to the manner in which it acts; experiments with serozyme have shown that solutions prepared according to his technic, contain fibrinogen; moreover, the failure of plasma, from which the so-called serozyme had been removed by treatment with tricalcium phosphate, to clot, was found to be due to the presence of alkali and phosphate. Thus the experimental basis for the conception of serozyme as a factor in coagulation, distinct from fibrinogen, ceased to be significant. Investigation of the nature of cytozyme and of the acetone-insoluble lipoids which it contains established the fact that the active lipoidal substances which possess the cytozymic action in the coagulation of the blood, combine with calcium with the liberation of acid. In addition, these lipoids form with protein an insoluble complex in the presence of a small amount of acid which by itself has little or no precipitating action upon protein without lipoid.

In the first experiments (1), (2), crude lipoidal extracts were used; then the active lipoidal substance was isolated in sufficiently purified form to suggest the chemical nature of the substance and the reaction. Finally, the last paper (3) recorded the results obtained with practically pure lecithin. Pure lecithin proved inactive but when hydrolyzed by heating with ammonia in the presence of calcium chloride a fraction was separated which possessed the coagulative properties of the active lipoid used in the previous experimental studies and reacted chemically to form a compound with calcium.

This paper records the results of a study made with cephalin, free from

[1] Presented at the meeting of the American Chemical Society, Division of Biological Chemistry, Cincinnati, Ohio, September 9, 1930.

lecithin and possessing a relatively high degree of purity, which was prepared from beef brain by a new method. In the experiments with this product, as in those with lecithin free from cephalin, it was possible to study not only its coagulative activity but also the chemical reaction between lipoid and calcium. Some preliminary experiments demonstrating reactions between protein and the purified lipoids were also made.

PREPARATION OF CEPHALIN. The ether-soluble fraction of beef brain insoluble in ethyl alcohol contains the major part of the cephalin of that tissue and the method usually applied (4) for its separation consists chiefly in repeated precipitation of the ether-soluble material with this alcohol. It was found in attempting to purify material obtained in this way that a large proportion of it was soluble in hot methyl alcohol and that a material of comparative purity, free from lecithin, could be easily isolated from this soluble fraction.

The membranes were removed from about 50 pounds of beef brains and the tissue was hashed to a pulp. The moist pulp was covered with acetone with which it was well mixed and allowed to stand over night. The acetone was then removed by filtration through Buchner funnels and the tissue spread before a fan for two or three hours. Drying was continued in a vacuum desiccator, and when dry, the material was pulverized. It was then extracted thoroughly with acetone followed by exhaustive extraction with 95 per cent alcohol. Thirty to forty liters of each of these solvents were necessary. (These extracts may be used for the preparation of lecithin.) The tissue was finally extracted with three 9-liter portions of ether containing 3 per cent of water, the extracts being separated from the tissue in a filter press between each extraction. The ether extract was concentrated to about one-third its volume and chilled below 0°C. over night. The white residue was removed by filtration in the ice box and the filtrate concentrated nearly to dryness *in vacuo* at 40°C. The residue was taken up in anhydrous ether with a trace of added water to facilitate solution and poured into from 5 to 10 volumes of nearly absolute ethyl alcohol. The precipitate was washed repeatedly with absolute ethyl alcohol by the use of a mechanical shaker until the extracts were practically colorless. The material was then dissolved in anhydrous ether as before, poured into boiling hot absolute methyl alcohol, and filtered through soft paper while hot. This operation was repeated once. The residue insoluble in hot methyl alcohol was discarded. The filtrate was cooled to room temperature and the precipitate was taken up in ether and reprecipitated from cold methyl alcohol and finally from acetone. The white acetone precipitate was separated in the centrifuge and dried *in vacuo* at from 56 to 57°C. When dry, the pulverized material was light yellow in color and crystalline in appearance.

Micro-analysis of cephalin

CARBON	HYDROGEN	PHOSPHORUS	TOTAL NITROGEN	AMINO-NITROGEN	RATIO P:N
per cent	*per cent*	*per cent*	*per cent*	*per cent*	
61.35	9.58	3.60	1.61	1.62	1:0.993

We are indebted to Mr. L. W. Hyman for all the chemical analyses with the exception of the amino-nitrogen determinations. The analytical results were obtained by the methods of Pregl, which, however, had to be so modified and supplemented as to require an investigation of the technical procedures.

THE REACTION OF CEPHALIN WITH CALCIUM CHLORIDE. When cephalin, prepared as described above, was treated with distilled water it emulsified easily and on sufficient dilution formed a clear solution. When dilute calcium chloride was added a precipitate was formed directly from the neutral mixture, the resulting suspension becoming distinctly acid. This precipitate was removed by centrifugalization and washed repeatedly with an equal mixture of 95 per cent alcohol and distilled water, the shaking machine and centrifuge being used. It was then shaken with absolute alcohol and finally treated with dry acetone. It was dried at 100°C. in an Abderhalden dryer and analyzed. The material was a creamy white bulky powder totally unlike cephalin in appearance. Chemical analysis of this product showed that one-half of a molecule of calcium was bound to the cephalin. The union appeared to be a chemical one since no trace of chlorides could be found. It would appear, therefore, that calcium chloride in aqueous solution reacts directly with cephalin to produce a calcium compound. The reaction of the mixture was definitely acid although no distinct end-point could be determined by titration.

It was also found that the calcium can be removed from the calcium-cephalin compound in the manner previously employed with calcium compounds of the active lipoid substance of tissue extracts and of the hydrolyzed fraction of pure lecithin. This method consists in simple shaking of the material with N/1 hydrochloric acid, followed by extraction of the lipoid with ether and washing the ether solution with distilled water.

Two- and four-gram portions of cephalin prepared as described were emulsified in two and four liters of distilled water. Equal volumes of N/20 calcium chloride were added and the precipitate was separated in the centrifuge. From 100 to 200 cc. of a 50 per cent mixture of 95 per cent alcohol and distilled water were added to the centrifuge bottle containing the precipitate and the mixture was shaken for ten minutes. It was then centrifugalized and the wash liquid discarded. This operation was repeated fifteen times. The precipitate was then shaken once with absolute alcohol and once with acetone. Final drying was carried out in the Abderhalden dryer at from 56 to 57°C. The analytical results obtained were as follows:[2]

Micro-analysis of calcium-cephalin compound

	CARBON	HYDROGEN	PHOSPHORUS	NITROGEN	CALCIUM	CHLORIDE	RATIO P:Ca
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Sample 1	61.88	9.66	3.61	1.55	2.39	0	1:0.515
Sample 2			3.65	1.61	2.46	0	1:0.522

The lipoid recovered from the calcium compound of cephalin contained 1.35 per cent total nitrogen and 3.40 per cent phosphorus.

[2] The fact that in the formation of the calcium-cephalin compound one molecule of calcium reacted with two molecules of cephalin lends support to the generally accepted conception of the structure of cephalin, according to which one of the hydrogen atoms of the phosphoric-acid group of the molecule is freely ionizable.

THE COMPARATIVE COAGULATIVE PROPERTIES OF CEPHALIN, OF CALCIUM-CEPHALIN COMPOUND AND OF THE RESIDUE AFTER REMOVAL OF THE CALCIUM. The cephalin prepared as described, the calcium compound prepared from it, and the residue of this compound after removal of calcium were examined parallel with a crude extract of the acetone-insoluble lipoids of heart tissue (cytozyme, Bordet) for coagulative properties. This was done by treating a defibrinated plasma possessing little or no coagulative action with the lipoid substances and observing the degree of activation resulting. Such a serum corresponds to the serozyme or prothrombin of Bordet and Delange.

REAGENTS. *Oxalated plasma.* The oxalated plasma was obtained by bleeding a rabbit from the carotid over paraffined surfaces into one per cent sodium oxalate, one part to nine parts blood, and removal of blood cells and platelets by prolonged centrifugalization at high speed, the usual method as previously described (5).

The calcified saline. The calcified saline was a 0.035 per cent solution of calcium chloride in physiological salt solution.

The prothrombin or serozyme solution. A few cubic centimeters of the oxalated plasma, poor in platelets, were recalcified and defibrinated. The serum was allowed to stand at room temperature over night. A serum prepared in this manner has little or no coagulative action by itself but after preliminary treatment with an active lipoid solution, in the presence of ionized calcium salts, acquires marked coagulative properties.

The dioxalate plasma. This strongly oxalated plasma was prepared by adding four volumes of 0.2 per cent sodium oxalate, in physiological saline, to one volume of the oxalated rabbit plasma.

The lipoid solutions. In preparing the lipoid solutions for testing, 10 mgm. of material were emulsified in 5 cc. of physiological saline free from calcium. The dilutions and amounts which were used are indicated in the protocols.

In the experiment of protocol 1, 0.02 mgm. of the crude tissue extract of the acetone-insoluble lipoids, when incubated with calcium chloride and prothrombin solution, developed distinct clotting activity. The incubation of calcium chloride and prothrombin alone gave rise to no such activity.

Similar results were obtained with cephalin, illustrated in protocol 2, but the amount of cephalin required to bring about a similar degree of clotting of the plasma was only 0.002 mgm.

When the calcium compound of cephalin was tested, no coagulation was obtained even when 0.2 mgm. of material was used, as indicated in protocol 3. After removal of the calcium, however, the residual material possessed coagulative activity almost comparable to the original cephalin: 0.004 mgm. were sufficient to clot the dioxalate plasma.

Protocol 1. The coagulative activity of the crude tissue lipoid (cytozyme, Bordet)

REAGENTS	QUANTITIES				
	cc.	cc.	cc.	cc.	cc.
Prothrombin solution	0.2	0.2	0.2	0.2	0.2
Tissue lipoid (cytozyme, Bordet)	0.1	0.0	0.0	0.0	0.0
Tissue lipoid, 1:10 (cytozyme, Bordet)	0.0	0.2	0.1	0.0	0.0
Tissue lipoid, 1:100 (cytozyme, Bordet)	0.0	0.0	0.0	0.2	0.0
Calcified salt solution, 0.035 per cent	0.2	0.2	0.2	0.2	0.2
Physiological salt solution	0.1	0.0	0.1	0.0	0.2
In water bath at 37°C.; 6 minutes					
Dioxalate rabbit plasma	0.1	0.1	0.1	0.1	0.1
Clotting time in water bath at 37°C.	1 min. 40 sec.	3 min. 15 sec.	8 min. 46 sec.	No clot	

Protocol 2. The coagulative activity of the purified cephalin free from lecithin

REAGENTS	QUANTITIES				
	cc.	cc.	cc.	cc.	cc.
Prothrombin solution	0.2	0.2	0.2	0.2	0.2
Cephalin solution	0.1	0.0	0.0	0.0	0.0
Cephalin solution, 1:10	0.0	0.1	0.0	0.0	0.0
Cephalin solution, 1:100	0.0	0.0	0.1	0.0	0.0
Cephalin solution, 1:1000	0.0	0.0	0.0	0.2	0.0
Calcified salt solution, 0.035 per cent	0.2	0.2	0.2	0.2	0.2
Physiological salt solution	0.1	0.1	0.1	0.0	0.2
In water bath at 37 C.; 6 minutes					
Dioxalate rabbit plasma	0.1	0.1	0.1	0.1	0.1
Clotting time in water bath at 37°C.	1 min. 23 sec.	1 min. 15 sec.	2 min. 18 sec.	No clot	

Protocol 3. The coagulative activity of the calcium-cephalin compound

REAGENTS	QUANTITIES			
	cc.	cc.	cc.	cc.
Prothrombin solution	0.2	0.2	0.2	0.2
Calcium-cephalin compound	0.1	0.0	0.0	0.0
Calcium-cephalin compound, 1:10	0.0	0.1	0.0	0.0
Calcium-cephalin compound, 1:100	0.0	0.0	0.1	0.0
Calcified salt solution, 0.035 per cent	0.2	0.2	0.2	0.2
Physiological salt solution	0.1	0.1	0.1	0.2
In water bath at 37°C.; 6 minutes				
Dioxalate rabbit plasma	0.1	0.1	0.1	0.1
Clotting time in water bath at 37°C.	No clot	No clot	No clot	No clot

Protocol 4. The coagulative activity of the cephalin residue after removal of the calcium from the calcium-cephalin compound

REAGENTS	QUANTITIES					
	cc.	cc.	cc.	cc.	cc.	cc.
Prothrombin solution	0.2	0.2	0.2	0.2	0.2	0.2
Cephalin residue after removal of calcium from calcium-cephalin	0.1	0.0	0.0	0.0	0.0	0.0
Cephalin residue after removal of calcium from calcium-cephalin, 1:10	0.0	0.2	0.1	0.0	0.0	0.0
Cephalin residue after removal of calcium from calcium-cephalin, 1:100	0.0	0.0	0.0	0.2	0.1	0.0
Calcified salt solution, 0.035 per cent	0.2	0.2	0.2	0.2	0.2	0.2
Physiological salt solution	0.1	0.0	0.1	0.0	0.1	0.2
In water bath at 37°C.; 6 minutes.						
Dioxalate rabbit plasma	0.1	0.1	0.1	0.1	0.1	0.1
Clotting time in water bath at 37°C.	3 min. 14 sec.	1 min. 29 sec.	1 min. 46 sec.	5 min. 10 sec.	No clot	

Howell (6), as early as 1910, and more recently Gratia and Levene (5) who worked with a purified product containing only 25 per cent of lecithin, concluded that cephalin rather than lecithin possessed the coagulative action, especially since pure lecithin was found to be inactive.

THE REACTION OF PURIFIED CEPHALIN AND LECITHIN WITH PROTEIN. The reaction of the purified cephalin and lecithin with protein was also studied in some preliminary experiments and results similar to those observed in the earlier work with protein and the crude extracts of the acetone-insoluble lipoids were obtained. Mixtures of albumin or of pseudoglobulin with cephalin were precipitated by a small amount of hydrochloric acid, which had no action upon either the protein or the cephalin alone. Furthermore, a similar precipitation occurred when calcium chloride was added to a mixture of cephalin and protein, doubtless due to the presence of acid liberated by the reaction between the cephalin and the calcium chloride.

A similar series of experiments was carried out with lecithin. When pseudoglobulin was mixed with lecithin, which, unlike cephalin, does not react with calcium chloride to liberate acid, no precipitation occurred upon the addition of calcium chloride. Moreover, when an amount of acid, equivalent to that used in the experiment with cephalin, was added to a mixture of pseudoglobulin and lecithin, no precipitation took place, indicating, as have all the results with calcium, the presence of a freely

ionizable hydrogen atom in the cephalin molecule and the absence of such an ion in lecithin.

The results of further study of these reactions will be published separately.

Discussion. The significance of these experimental studies of the coagulation of the blood should be apparent. Hitherto research has revealed certain conditions under which the phenomena take place. The influence of contact, the action of the platelets or extracts of tissues and that of the calcium salts have been detected and to a degree differentiated. The hypothetical suggestion of an additional factor in the nature of an enzyme, however, has been considered essential.

In order to account for this enzyme, thrombin, which the serum of freshly clotted blood contains, it has been necessary to postulate the existence in the circulating blood of either precursors or inhibiting substances. The inhibiting action of heparin on coagulation has been demonstrated by Howell. The precursors of thrombin have not been demonstrated.

Bordet worked with distinct preparations of different fractions, or components, of the blood with which the serozymic and cytozymic activities were associated, and these were assumed to react with each other to form thrombin. In our earlier experiments, the serozyme solutions were found to contain fibrinogen. In these, as well as in later studies, the cytozymic substances reacted with calcium chloride, with the liberation of acid, irrespective of whether the crude extracts of tissue, the di-glyceride phosphoric acid isolated from them, the hydrolyzed product of pure lecithin, or purified cephalin was used. The chemical reactions of the calcium salt have been demonstrated with each of these lipoidal substances in the state in which they possess the coagulative activity. The calcium compound washed free of acid has no coagulative action for blood plasma whereas fibrin is formed when cephalin and calcium chloride are added directly to plasma. Lipoid and protein form an insoluble complex in the presence of a trace of acid which has no apparent effect upon the protein without lipoid or upon the lipoid alone. They likewise precipitate from solution in the presence of calcium chloride which, in reacting with the lipoid, liberates acid.

Coagulation proceeds from the surface of contact not only after the blood has been shed in vessels but also in the tissues, as strikingly illustrated by the fibrinogenesis in a pneumonic exudate. The physical characters of the substances involved must be determining factors in the actual formation of the clot. Thus, as suggested in an earlier paper, the active lipoid, which is set free from the cells after blood is let and which is strongly adsorbed at wettable surfaces, doubtless reacts with ionized calcium salts and protein in a manner similar to the reactions which have been demonstrated with cephalin, calcium chloride and protein.

SUMMARY AND CONCLUSIONS

A new method for the preparation of a relatively pure cephalin, free from lecithin, was developed.

Purified cephalin prepared by this method possessed a high degree of coagulative activity.

An aqueous solution of cephalin reacted with calcium chloride in neutral solution to produce a distinct chemical compound of cephalin with calcium.

The calcium compound, when washed free of its reaction product, hydrochloric acid, was inactive as a coagulant of blood plasma.

The cephalin recovered from the calcium compound possessed a high degree of coagulative activity.

When an aqueous solution of cephalin was mixed with a solution of serum albumin or pseudoglobulin in the presence of a slight amount of acid (HCl), a globulin-like precipitate was formed whereas in the absence of acid no visible reaction occurred. No precipitation occurred when this amount of acid was added to the cephalin or the protein alone. Similar results were obtained when calcium chloride instead of acid was added to a mixture of cephalin and serum protein.

Lecithin, on the other hand, which has no coagulative action, did not give rise to precipitation when mixed with these proteins either in the presence or absence of similar amounts of acid or of calcium chloride.

The results of this study indicate that cephalin, like the active lipoid fractions isolated from the crude tissue extract and from pure lecithin, coagulates blood plasma and, further, that this action is associated with a chemical reaction of the lipoid with ionized calcium salts resulting in the formation of mineral acid.

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OVARIAN HORMONE AND METABOLISM

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In studying the relation of ovarian hormone to metabolism it is important to note what hormone is being considered. There seem to be three hormones in the ovary and the data on normal women would be influenced by these three hormones collectively. In the assay and injection experiments, the *follicular* hormone was the one used because at the time the work was done it was the only one we had means to assay or we could obtain. It had been repeatedly reported by Frank and others that the follicular hormone occurs in the blood of women in the pre-menstrual period and is absent or deficient in the post-menstrual period, and we have confirmed this finding to the extent that some samples of 40 cc. of pre-menstrual blood produced an abundantly cornified vaginal smear in an ovariectomized mouse, whereas all samples of 40 cc. of post-menstrual blood failed to do this. After 400 days of work on the assay of the follicular hormone, we ruled out all results unless there was a *great abundance* of cornified cells in the vaginal smear. (It seems to us more logical to take out the ovary from the dorsal side without cutting into the peritoneal cavity.)

Laqueur, Hart and deJongh (1926) reported an increase in the basal metabolic rate of ovariectomized mice on injection of the follicular hormone. McClendon and Burr (1929) reported a slight rise in basal metabolic rate of a woman who apparently had been devoid of ovarian function since birth (absence of menstruation and puberty-growth), on the injection of 1000 or more mouse-units of follicular hormone during a day. We have repeated this experiment on another woman who had never menstruated although she was thirty-five years old. She was also sterile and had only vestiges of nipples. On injecting 30,000 mouse units of follicular hormone in oil (which was given to us by Doctor Laqueur) there was a rise in basal metabolic rate during the day and it continued high the second day. Although Laqueur claimed the potency was as great as that of Doisy's crystals of "theelin," since it was dissolved in oil we did not consider it pure. We obtained some pure "theelin" from Doctor Kamm but were not able at the time to find a proper subject for the metabolism experiment. We therefore injected this at the rate of 50 units every half-hour for

ten doses into a man and determined the basal metabolic rate every half-hour. There was no consistent rise in metabolism with the accumulation of the hormone. This is the same result as reported by Laqueur for male mice and normal female mice and by Kunde, D'Amour, Carlson and Gustavson (1930) for normal female dogs. It is not clear why the hormone should raise the basal metabolic rate of ovariectomized females or those with congenital lack of ovarian function and not that of normal females or males. Certainly the males show lack of ovarian function. One might suppose that the rise in basal metabolic rate was due to the growth of the endometrium, but time was not sufficient for very much growth, and, according to Corner and Hisau, the *follicular* hormone does not cause a growth of the endometrium. We are forced to leave these injection experiments in this unfinished state and pass on to the cyclical changes in normal women.

Since the follicular hormone increases in the blood during the intermenstrual period, if it caused an increase in basal metabolic rate, there should be such an increase in metabolism during this period. Benedict and Finn made such a study on one woman and we have averaged the cubic centimeters of O_2 per minute for the pre-menstrual and for the post-menstrual weeks. For the post-menstrual weeks there were 178.8 cc. of O_2 per minute and for the pre-menstrual weeks 179 cc. of O_2 per minute. The slight increase indicates that statistical methods must be used to find out its significance. A number of writers have reported a greater increase. Some of them are Snell, Ford and Rowntree (1920), Rowe and Eakin (1921), Wakeham (1923), Hafkesbring and Collett (1924), Collett and Liljestrand (1924), and Conklin and McClendon (1930). On the other hand a number of writers have denied this, the latest being Sandiford, Wheeler and Boothby (1931). The data of the latter show a slight rise, however, the average of the pre-menstrual week being slightly higher than the post-menstrual week. The average for the post-menstrual week is 32.9 ± 0.2 and for the pre-menstrual week 33.3 ± 0.2. This careful work combined with that of Benedict and Finn (1928) recalls the necessity of statistical methods. We have made prolonged series of determinations on twenty-three women, varying in age from nineteen to thirty-five years, with a mean of 21.2 years. The average of the post-menstrual period was 32.5 calories per square meter per hour and for the pre-menstrual period was 33.7 calories per square meter per hour. The grand average of the basal metabolic rate was 33.6 calories per square meter per hour, which corresponds closely to the average given by Sandiford, Wheeler and Boothby (1931) for a normal woman during her 34th to 41st years, which is 33.1 ± 0.06. It should be noted that these are trained individuals and cannot be used as standards for clinical work.

We believe the spread of the curve and the probable errors will be more

evident if results are put in percentages of the mean values of these women. The basal metabolic rate during the post-menstrual period for these twenty-three women was 98.88±0.15, and for the pre-menstrual period 101.41±0.27. The determinations were as follows: Post-menstrual period, 82 (once), 87 (once), 88 (once), 90 (four times), 91 (twice), 92 (5 times), 93 (3 times), 94 (12 times), 95 (12 times), 96 (11 times), 97 (17 times), 98 (14 times), 99 (18 times), 100 (8 times), 101 (19 times), 102 (16 times), 103 (11 times), 104 (4 times), 105 (4 times), 106 (7 times), 107 (4 times), 108 (twice), 109 (twice), 110 (once), (n = 179); pre-menstrual period, 86 (once), 88 (once), 90 (once), 91 (once), 92 (once), 93 (twice), 94 (4 times), 95 (7 times), 96 (9 times), 97 (8 times), 98 (11 times), 99 (11 times), 100 (12 times), 101 (18 times), 102 (9 times), 103 (11 times), 104 (12 times), 105 (14 times), 106 (8 times), 107 (6 times), 108 (5 times), 109 (twice), 110 (twice), 111 (twice), 112 (3 times), 116 (once), 120 (once), (n = 163). The difference between the post and pre-menstrual periods is a rise of 2.53 per cent and the probable error is 0.15 for the post and 0.27 for the pre-menstrual period so we have here a significant rise in the metabolic rate during the inter-menstrual period, based on 342 determinations. These individuals were not selected except that only women who would thoroughly coöperate and who did not show nervousness or complain of menstrual pain, were utilized. The average number of determinations on each woman was less than in the work of Conklin and McClendon and the difference between the post and the pre-menstrual periods was not quite so great but the probable errors were very much smaller owing to the larger number of determinations.

Since it has been shown by Frank (1929) Steinach (1925) Hartman (1926) and others, that the ovarian hormone causes an increase in the mammary gland and nipple, an attempt has been made to study the volume of the nipple in the post and pre-menstrual periods. In this case the volumes of the nipples are recorded in per cent of the average volumes for the women studied. The volumes during the post-menstrual week measured on thirteen women were 93.9±0.693, and the volumes during the pre-menstrual week were 106.3±0.8. The individual determinations were of the post-menstrual period: 82 (once), 84 (once), 85 (once), 86 (3 times), 88 (twice,) 89 (twice,) 90 (once), 91 (once), 92 (4 times), 93 (once), 94 (3 times), 95 (3 times), 96 (once), 97 (twice), 98 (once), 99 (3 times), 100 (once), 101 (twice), 102 (twice), 103 (once), 108 (once), (n = 37) and for the pre-menstrual period: 93 (twice), 96 (once), 98 (once), 100 (once), 101 (twice), 102 (twice), 103 (4 times), 104 (6 times), 106 (twice), 107 (4 times), 108 (once), 109 (3 times), 110 (twice), 111 (3 times), 118 (once), 119 (twice), 120 (once), 121 (once), (n= 39). The increase in volume of the nipples from the post to the pre-menstrual week was 12.8 per cent, and is many times larger than the probable error.

SUMMARY

We have studied the period between menstruations on the hypothesis that the changes are due to changes in concentration of hormone and find a slight rise in basal metabolic rate. Owing to the fact that this rise is less than the daily variations, a large number of determinations (342) had to be made in order to reduce the probable error. We found an increase in the volume of the nipples during the same period. Since it is well known that the endometrium increases during this period, it seems possible that the rise in basal metabolic rate may be due to the growth of tissue in the endometrium and mammary glands. As to what hormones are concerned with these changes, we have no data of our own except that the report by McClendon and Burr that injection of follicular hormone increased the basal metabolic rate of a woman lacking ovarian functions, was confirmed on another similar woman, but there was no quantitative relation between the amount of the hormone injected and the rise in B.M.R. It seems probable that a hormone from the corpus luteum causes the changes we have found, or that they are due to more than one hormone.

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THE PENETRATION OF LIGHT THROUGH HUMAN SKIN[1]

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In previous publications (Bachem and Kunz, 1929) (Bachem and Reed, 1929, 1930) it has been shown that the different methods of measuring transmission of rays through tissues have given widely varying results due to scattering and fluorescence. Therefore a method has been developed by which penetration could be studied under conditions very similar to those for artificial and natural irradiation. Lately this method has been employed in extending our studies to the far ultra-violet and to the infra-red regions.

The principle of the method as previously described depends on filtering the rays of a Kromayer lamp, an incandescent bulb or an infra-red radiator through a combination of filters that will limit the range to a narrow band. Figure 1 shows the combinations used together with the range and percentage intensity of the various bands. The heading numbers in this chart refer to Wratten gelatine filters. In addition we used a filter of uviol glass, and one of Corning glass, also a quartz cell filled with chlorine-bromine gas.[2]

As will be explained later, the spectrograph is not a reliable instrument for measuring transmission through skin. Therefore an elaboration of the method of Stenstroem and Reinhard (1926) was developed. This consisted of exposing a photographic plate on a holder with a screen in which was an opening 15 mm. in diameter. This was placed at right angle to the beam from the lamp. The filter was then inserted and the plate moved for exposure on a new field. The skin specimen mounted on a composition tube was then placed in the opening of the screen so that it was resting practically against the surface of the plate. This position assured full registration of scattered rays. A schedule of time exposures was then followed which permitted accurate application of the Schwartzschild factor to the readings obtained on the Martens photometer. By this method duplicate readings on the same specimen did not show variations

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[2] One of these was loaned by the Hanovia Company.

LAMP	INF. RED RADIAT.	INCANDESCENT LAMP		WATER COOLED QUARTZ MERCURY ARC (KROMAYER)							
FILTER	NO	88a 7 mm WATER	23e 1.5mm WATER	16 2	75η	36 2	18a 2	18a UVIOL	18a 12	VAPOR CORNING L.AGET.	2 VAPOR CORNING
>1400	!	0	0								
>1000	t	!	t								
>700	0	t	!	—							
578				60	0						
546				60	1						
491				0	18	0					
435					0	25					
405						15	0				
391						3	6	0			
38:						0	5	7			
365							0	20			
35:								22			
34:								11	0		
334								5	2		
313								0	2		
302									0		
297										0	
293										1	
289										2	
285										3	0
280										5	2
275										2	2
270										0	3
267											3
265											2
263											2
260											1
257											1
253											0
Av λ	1000 -5000	750- 1400	700- 1200	560	491	420	385	360	324	280	267

Fig. 1. Schedule of filters used. Heading figures refer to Wratten gelatine filters. Column figures refer to range and percentage intensity of the various spectral regions.

of more than ±5 per cent. With the shorter exposures the accuracy was much greater. When the infra-red region was investigated a similar arrangement was employed except that the skin was placed in direct con-

tact with the bolometer of the Burt photometer. The direct results are shown in figure 2.

Average figures from this chart were used to plot transmission curves for the stratum corneum, the epidermis, and the skin with the whole corium included. Figure 3 shows these curves.

Skin specimens from three sources were used; 1, cantharides blisters on the flexor side of the forearm; 2, strips from the ventral abdominal wall of fresh autopsy material shaved with a microtome, while frozen, to the desired thickness; 3, tensed folds of the scrotum of living subjects, and scrotal strips from dead subjects Except in the latter case, when calipers

SPECIMEN THICKNESS mm			CANTHARIDES BLISTER CORNEUM			CANTH. BLIST. EPIDERMIS GERMINAT.		SKIN .5-1.5‡ EPIDERMIS + PAPILLARE		SKIN 1.7-2.3 EPIDERMIS + CORIUM		GROIN DEAD LAYER 2		LIVE LAYERS 4	SKIN + FAT 9	13	25
LAMP	FILTERS	λ	.03	.03-.03+		.05-.05+											
INFRA RED	NO	1400+	45	45	42	30	25	15	13	9	7	—	—	6	3	1	0
INCAN-DESCENT BULB	7mm WATER 88a	1000+	70	72	70	60	52	33	23	20	15	40	17	15	9	5	0
	15mm WATER 23e	800+	77	77	70	65	60	37	32	25	19	50	28	25	10	8	1
MERCURY ARC	16 2	560	85	90	87	80	74	39	30	8	3	—	—	3	2	1	0
	75 η	491	85	88	84	77	74	28	16	6	2	—	—	—	0	0	0
	36 2	420	80	85	81	70	50	10	7	3	1	—	—	—	—	—	—
	18a 2	385	80	80	75	62	46	6	1	t	0	—	—	—	—	—	—
	UVIOL 18a	360	70	78	73	50	47	3	1	0	0	—	—	—	—	—	—
	18a 12	324	54	60	56	30	24	t	0	0	0	—	—	—	—	—	—
	VAPOR CORNING LEAD ACET.	280	25	20	16	13	11	0	0	0	0	—	—	—	—	—	—
	2 VAPOR CORNING	267	20	18	15	9	6	0	0	0	0	—	—	—	—	—	—

t = trace

Fig. 2. Percentage transmission in all specimens for the various spectral regions. Column figures represent percentages of applied intensity transmitted.

were used to measure it, the thickness of the various specimens was subsequently determined by means of a microscopic stage micrometer.

On account of the width of the filter spectra the finer details in the far ultra-violet and infra-red were not registered. Therefore, the curves (fig. 3) have been corrected in the ultra-violet by comparison with the absorption curves obtained in the previous spectrographic experiments. The hemoglobin absorption at 400 and between 500 and 600 mμ shows up in the curves, but probably not intensely enough, due to the width of the filter spectra, and on account of working with dead material, moistened by saline solution.

From these observations the distribution of the transmitted light and its

absorption in the various parts of the skin was determined. Figure 4 gives the results for the most important wave lengths between 200 and 1400 mμ.

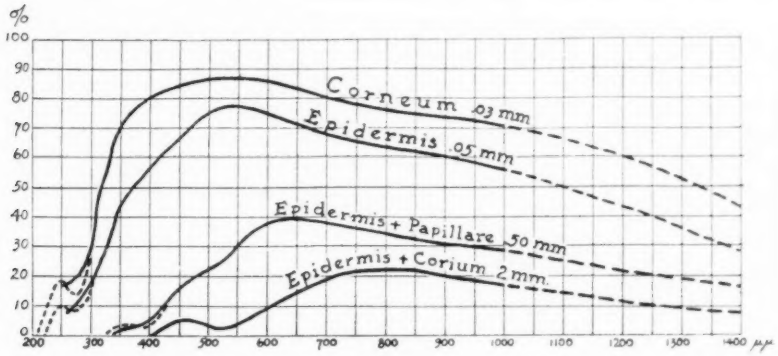

Fig. 3. Transmission curves for the various skin layers

LAYER	λ / mm	200	250	280	300	400	550	750	1000	(1400)	μμ
		100	100	100	100	100	100	100	100	100	APPLIED
CORNEUM	.03	(100)	(81)	(85)	(66)	(20)	(13)	(22)	(29)	(56)	ABSORBED AND REFLECTED
		0	19	15	34	80	87	78	71	44	TRANSMITTED
+MALPIGHII	.05	(0)	(8)	(6)	(18)	(23)	(10)	(13)	(6)	(16)	ABSORBED
		0	11	9	16	57	77	65	65	28	TRANSMITTED
+CORIUM	.20	(0)	(11)	(9)	(16)	(56)	(72)	(44)	(48)	(20)	ABSORBED
		0	0	0	0	1	5	21	17	8	TRANSMITTED
+SUBCUTAN.	.25	(0)	(0)	(0)	(0)	(1)	(5)	(20)	(17)	(8)	ABSORBED
		0	0	0	0	0	0	1	0	0	TRANSMITTED

	EXTREME U-VIOL.	FAR ULTRA VIOLET	NEAR U-VIOL.	VISIBLE VIOL GREEN RED	NEAR INFRA RED	FAR INF RED
GENERAL REMARKS	All absorbed by corneum. No radiation reaching germinativum.	Greatest absorption in stratum corneum. Some radiation reaches corium (papillare). No radiation reaches subcutaneous layers.	Relatively large absorption in stratum Malpighii.	Minimum absorption in stratum corneum. Most radiation absorbed in corium. Pronounced radiation reaching subcutaneous layers	Strongly increasing absorption in upper layers, decreasing in lower layers.	Practically no penetration

Fig. 4. Light distribution in the layers of the skin. The number 100 designates the applied intensity of the light, regardless of the source. The encircled numbers then represent percentages absorbed in each layer. The numbers in the narrow zones between layers represent the percentages of the original intensities transmitted through the layer above.

The average thickness of the stratum corneum was 0.03 mm. from our observations on the flexor side of the arm and the abdominal skin. The

average thickness of whole epidermis was estimated as 0.05 mm.; for the epidermis plus the corium 2 mm., and 25 mm. for the skin plus the subcutaneous layers, in which practically every trace of light disappears.

The applied light is arbitrarily expressed as 100 for each wave-length. The simple figures in the horizontal lines between the various layers indicate percentage of light transmitted through the layer above, and entering the layer below. The light absorbed in the respective layers is given in encircled figures. A certain inaccuracy occurs however in the figures for the upper layers which reflect a small part of the light. This inaccuracy is indicated by vertical arrows; the actual absorption of light in these layers will be somewhat smaller.

The question whether the wave-length of the rays remains the same on passing through the skin or whether it is changed by fluorescence into longer waves was investigated by two methods:

1. The relative position of the skin specimen and filter toward light and plate was reversed; in case of skin fluorescence the registered intensity would be diminished as compared with the original position. No measurable difference was observed, however.

2. An additional filter was inserted between skin and plate which would absorb the light transmitted through the first filter, but would transmit fluorescent light of longer wave-length. The trace of light transmitted was too indefinite to permit any conclusion.

As mentioned above, spectrographic measurements of light transmitted through skin are not reliable. This need not be emphasized further than to cite some figures obtained by other investigators for the wave-length $\lambda 289$. Hasselbalch (1911) reported transmission through 0.1 mm. thickness of skin of only 0.01 per cent, as determined by the spectrograph; Macht and co-workers (1928) reported for approximately the same region, transmission of 6 to 10 per cent through rabbit skin 1.2 mm. thick. For a thickness of 0.1 mm., of course, the calculated percentage would be about 80 per cent. For this spectral region we have found by our method transmission of approximately 10 per cent, through a specimen 0.1 mm. thick.

While successive readings on the same specimen agree closely it cannot be expected that readings on two different specimens of the same thickness, even from the same subject, will agree so closely when one considers that differences in chemical composition, particularly pigment content and retained hemoglobin, relative thickness of the various layers, physical structure, and artifacts due to handling and treatment all play a part in determining transmission of rays. The possible relation of such variations in transmission to light sensitivity of the skin is the subject of a further investigation not yet completed.

From these results the following generalizations may be made:

1. Visible and near infra-red rays are strongly absorbed by the blood of the corium and subcutaneous layers, with direct effects upon the blood to be expected. The heating effect must be rather mild on account of the small absorption in the epidermis and the strong convection by the blood stream.

2. The far infra-red has very little penetrating power. Most of it is absorbed in the epidermis. The heat produced is slowly conducted away by the blood stream and by the air. Over-exposure of the germinal layer may lead directly to blistering.

3. There is greater variation in percentage penetration of ultra-violet than in other parts of the spectrum. At 280 mμ the absorption in the corneum and prickle-cell layers is very pronounced. The great anti-rachitic effect of this wave-length must occur in or about these layers. On both sides of this band, near 300 and 250 mμ the penetration is greater, more radiation reaching the stratum Malpighii and the corium. These observations, together with those of Hausser and Vahle (1921) indicate that erythema production occurs in the germinal layer (or the corium) under the shadow of the upper layers. The stratum corneum and granulosum must play an important rôle in the light protection of these sensitive layers. From 250 mμ down the absorption in the horny layer increases rapidly. Near 200 mμ the absorption is so complete as to prevent any radiation from reaching the living layers of the skin.

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RESPIRATORY AND CARDIO-VASCULAR CHANGES IN THE CAT DURING CONVULSIONS OF EXPERIMENTAL ORIGIN[1]

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In a previous paper (Coombs, Wortis and Pike, 1931), it was pointed out that the participation of the autonomic system in epileptic seizures has been little investigated. The multifarious papers on the Aschner-Dagnini reflex, the reaction to pilocarpine and the like (Crouzon, 1929; Pagniez, 1929) have given little information about what actually occurs in a convulsion. The task of devising an explanation of a process can be completed only when we have access to knowledge of the kind and order of events in the process itself. The present series of experiments was directed toward a study of the reactions of the cardio-vascular system in convulsions of experimental origin. In addition, the changes in respiratory rhythm and amplitude have been recorded simultaneously with the blood pressure tracing.

Francois-Franck (1878) showed that a marked rise of blood pressure occurs in convulsions arising from electrical stimulation of the motor area of the cerebral cortex and Muskens (1928, p. 244) states that, in epilepsy in the human, the blood pressure is highest when the clonic convulsions are most rapid. On the other hand, one finds the statement in the literature of pharmacology (Sollman, 1917) that camphor may bring about a fall of blood pressure due to peripheral action on the blood vessels. Camphor monobromide and oil of absinth, the active principle of which is regarded as an isomer of camphor, have been extensively employed for the experimental production of convulsions (Wortis, Coombs and Pike, 1931). Muskens (1928, p. 10) remarks that the fit produced by camphor is capable of comparison with a genuine epileptic fit. It seemed of interest to determine:

A. Whether convulsions can be induced when conditions are made such that the systemic blood pressure will fall.

B. Whether the blood pressure rises or falls in intact animals during experimentally induced seizures.

[1] The experiments upon which this paper is based, with the exception of the control experiments mentioned incidentally, were done in the Physiological Laboratory of the New York Homeopathic Medical College.

C. The cardio-vascular response to the intravenous injection of camphor monobromide or absinth in curarized animals.

EXPERIMENTAL PROCEDURES. The technique of the injection of absinth has been described elsewhere (Pike, et al., 1929; 1930). We have used both absinth and camphor monobromide in these experiments. In addition, a cannula inserted in the femoral artery was connected with a mercury manometer and a continuous record of blood pressure was taken, while a stethograph placed at the level of the diaphragm was connected with a tambour which recorded respiratory movements.

In some experiments where transection of the spinal cord was done, cats were anesthetized with ether during the operation and the animals then allowed to recover from the effects of the ether for from one to three hours before further procedures were carried out. Transection of the spinal cord was done for two reasons:

1. To immobilize the hind legs so that the cannula in the femoral artery should not forcibly be pulled loose during the convulsive seizure.

2. To eliminate more and more the possible element of the activity of the skeletal muscles upon the blood pressure response by making the transection as high as the level of the second or third thoracic segment.

When curare was injected, no transection of the spinal cord was made and artificial respiration was given by means of a tube introduced into the larynx after the effects of the curare became apparent. In one case, the blood pressure cannula was tied into the carotid artery, exposed under ether, after which the wound in the neck was freely swabbed with novocaine. The abdominal aorta could then be compressed just below the diaphragm without interrupting the blood pressure record.

THE EXPERIMENTAL RESULTS. *A. The cardio-vascular and respiratory changes after transection of the spinal cord.* 1. When the transection is made high up, at the level of the second or third thoracic segment, there is a fall of blood pressure when absinth or camphor monobromide is injected, the degree of which is related to the quantity of drug injected. The respiratory movements increase in rate and amplitude. Clonic twitches appear about the head and in the fore limbs when the minimal convulsive dose is reached, and give way to tonic extension as the lethal dose is approached. Struggles and cries may occur before the onset of convulsions, just as in control animals. Pupillary dilatation and salivation may also occur, but the pupil does not become insensitive to light until the convulsive dose is reached.

Under the conditions of high spinal transection, clonic twitches are sometimes wholly absent, and tonic extension with death occurs at the first dose which is effective as a convulsant.

The fall of blood pressure, once started, may continue until it reaches a level from which no recovery will occur unless some remedial measures

are taken, and the animal will die at a dose which is far below the lethal, and even below the minimal convulsive dose in control animals. It is a matter of some importance to note that a convulsion may occur with either

Fig. 1

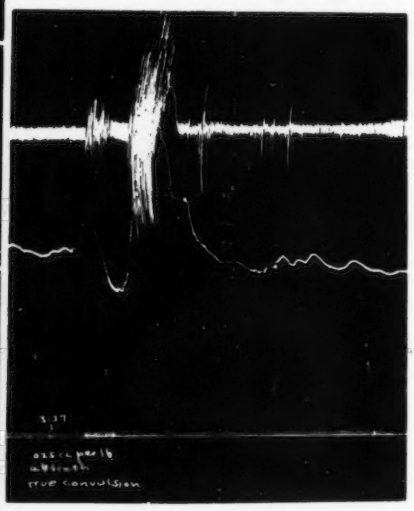

Fig. 2

Fig. 1. Shows the effect of the injection of the minimal convulsive dose of camphor monobromide upon respiration (upper tracing) and blood pressure, in a cat in which the spinal cord had been transected at the fifth thoracic segment. Base line at signal. The sudden increase in amplitude of the respiratory tracing indicates the onset of the clonic convulsion. The blood pressure falls during the early stage of the convulsion, and rises slightly at the time when the upper tracing shown the greatest amplitude of movement. There is a less violent secondary convulsion with a slight rise in blood pressure shown at the right of the figure.

Fig. 2. Shows the effect of the injection of absinth on the respiration and blood pressure of a cat without lesion of the spinal cord. The preliminary fall of blood pressure becomes a marked rise at the time of the most violent muscular activity of the clonic convulsion. Base line at signal.

absinth or camphor monobromide while the blood pressure is falling. The graphic record (fig. 1) of one of the convulsive seizures in a typical experiment illustrates this. The protocol of the experiment from which this record is taken, follows.

November 9, 1930. Male cat, weight 8¼ pounds.

2:25 p.m. Ether, spinal cord transected at 5th thoracic.

2:35 Ether intermitted, cannula in femoral artery for blood pressure record.

3:00 Camphor monobromide, 0.012 cc. per pound; total 0.10 cc. A true clonic convulsion followed; pads of fore feet glistened; rapid respiration, profuse salivation.

3:09 Camphor monobromide, 0.009 cc. per pound; total 0.08 cc. Twitches, respiratory effect, blood pressure rose instead of falling, but no true convulsion followed.

3:19 Camphor monobromide, 0.008 cc. per pound; total 0.07 cc. followed at 3:19:30 by 2 cc. adrenalin, 1:50,000. Blood pressure began to fall: no clonic movements; then, as blood pressure rose, a true clonic convulsion followed.

3:32 Camphor monobromide, 0.015 cc. per pound; total 0.12 cc. No true clonic convulsion, only twitches and restlessness.

3:42 Camphor monobromide, 0.018 cc. per pound; total 0.14 cc. True clonic convulsion, with falling blood pressure.

3:45 Another clonic convulsion.

3:52 Camphor monobromide, 0.020 cc. per pound; total 0.16 cc. convulsion with much tonic extension, delayed in appearance until 3:54. Asphyxial movements of hind limbs, blood pressure very low, and respiration failing.

3:58 2 cc. of 1:10,000 adrenalin. Respiration improved.

4:08 Camphor monobromide, 0.022 cc. per pound; total 0.18 cc. washed through with saline containing a trace of adrenalin. A true clonic convulsion, followed after two or three minutes by asphyxial extension of hind limbs and respiratory gasps.

4:15 Death.

Post-mortem. Thymus small, stomach slightly dilated, few raised whitish spots, 3–4 mm. in diameter on lungs, other organs appear normal.

The intravenous injection of a commercial preparation of adrenalin will frequently bring about a restoration of the blood pressure and a recovery of the animal. Such an injection immediately following a dose of absinth or camphor monobromide which would not be effective under the conditions of low blood pressure attending high spinal transection, may be, and usually is, followed by a clonic convulsion when the systemic blood pressure rises. This is in agreement with some earlier observations on control animals (Pike and Notkin, 1930). Since the action of adrenalin is of such short duration, ephedrine was also used to restore blood pressure. It is effective for a much longer time than adrenalin—one cubic centimeter of 2 per cent aqueous solution being adequate to maintain the blood pressure at a high level for more than half an hour following its injection. A dose of absinth or camphor monobromide which is just below the minimal convulsive becomes sufficient to elicit good clonic convulsions following its administration, just as with adrenalin, and this effect may be repeated, owing to the high level at which the blood pressure is held. Its injection, however, following the lethal dose of absinth, when respiration has reached

the stage of the terminal gasps, and blood pressure has fallen, does not resuscitate the animal. This ineffectiveness of intravenous injection of adrenalin after absinth or camphor monobromide is paralleled by a similar situation after repeated successive occlusions of the head arteries, where, when recovery is slow and blood pressure tends to remain well down, intravenous injection of adrenalin is followed by a prompt rise of blood pressure and an acceleration of the process of recovery. But if occlusion of the head arteries is now repeated, a condition is reached in which injection of the same dose or even larger doses of adrenalin is ineffective. There may be only a slight transient rise of blood pressure with no return of respiratory activity.

There is a question whether the recovery of the animal or the warding off of its death when adrenalin or ephedrine is administered, is due solely to its action upon the peripheral blood vessels and the heart, or to some other effect. We have accordingly tried maintaining the blood pressure during the convulsive period by mechanical compression of the descending aorta. The blood pressure cannula was tied into one carotid artery, as previously described, and the aorta compressed by pushing the thumb of one hand inward just below the lowest rib until the furrow on the ventral surface of the vertebral column could be felt. Downward pressure of the thumb compressed the aorta. The effect of the compression was tested during an interval between the earlier injections of absinth. The spinal cord had been transected at the level of the fifth thoracic vertebra.

A moderate dose of absinth—0.013 cc. per pound—was followed by acceleration of the respiratory rhythm, struggles and fall of blood pressure almost to base line, with failure of respiration. The injection of 8 cc. of 1:50,000 of adrenalin was followed by a prompt recovery of the animal. This procedure was repeated with a slightly larger dose of absinth, 0.019 cc. per pound and 10 cc. of the adrenalin solution, with subsequent recovery. There were no true clonic convulsions following either dose of absinth, although either would have been lethal if the blood pressure had not been restored by adrenalin. After an interval, a third dose of absinth—0.019 cc. per pound—was injected and washed into the circulation by 10 cc. of 0.9 per cent sodium chloride solution injected through the same hypodermic needle in the femoral vein. After a delay of about 30 seconds, to allow the absinth to be distributed throughout the circulation, the abdominal aorta was compressed for a period of four and one-half minutes, during which time the blood pressure remained well up. There were cries and other evidences of excitation due to absinth. When the compression of the abdominal aorta was intermitted, the blood pressure fell to base line and respiration ceased. The injection of adrenalin and saline was ineffective in restoring cardio-vascular activity.

At post mortem, the stomach was only moderately distended. In con-

trol animals, the dilatation of the stomach is often extreme. The thymus was large, although the cat weighed $7\frac{1}{4}$ pounds. We may mention in passing that, in a control series, all animals in which a large thymus or a large spleen or both, were found post mortem, showed a greater susceptibility to absinth, as measured by the lethal as well as the minimal convulsive dose, than other animals. These results will be presented elsewhere. There were old lacerations of the free surface of the spleen, with adhesions of the omentum. The left adrenal had been slightly crushed by the pressure of the thumb over the descending aorta. This is an accident which has been infrequent in our experience.

2. When the transection of the spinal cord is made at lower levels—seventh thoracic or below—the results are somewhat different. The fall of blood pressure due to transection is less, since the heaviest outflow of vasomotor fibers is through the sixth thoracic root (Langley, 1892; Ranson and Billingsley, 1918; Winkin, 1922). The intravenous injection of absinth or camphor monobromide is attended by a preliminary fall in blood pressure, but this fall does not extend to the base line, and recovery to the original level, except after the lethal dose, is spontaneous.

With the appearance of clonic convulsions, there is usually a sharp rise of blood pressure, the magnitude of which bears a general relation to the intensity of the muscular contractions. The respiratory acceleration is marked, especially before the onset of the clonic convulsions, and the other phenomena of the convulsion appear with more certainty than when the transection of the spinal cord is at a high level.

B. The cardio-vascular and respiratory changes in intact animals. These differ only in degree from those seen after transection of the spinal cord at the level of the seventh thoracic segment or lower (fig. 2). There is the same preliminary fall of blood pressure after the injection of absinth or camphor monobromide, the amount of the fall depending upon the amount of the dose. There is generally a sharp rise of blood pressure with the onset of the clonic convulsions, or even of the single muscular twitches which follow small doses. Dilatation of the stomach is much more frequently observed post mortem in animals with a low transection of the spinal cord or in control animals than in those with a high transection.

Following each convulsive seizure, there is a drop in the mean blood pressure level which is not regained during the period before the next injection. This is the case with absinth and camphor monobromide alike. The drop in mean blood pressure is not marked when doses below the minimal convulsive are given. But with the appearance of the convulsive seizures, mean blood pressure declines more sharply in the intervals between convulsions. This may be a vascular condition similar to that observed by one of us (Coombs, 1926) in the intervals between the periods of temporary ligation of the cerebral arteries. There is the same steady lowering of the

mean blood pressure in successive intervals between occlusions of the head arteries. The following tabulation, taken from our blood pressure records, illustrates this.

TABLE 1

MEAN BLOOD PRESSURE BEFORE	FIRST DOSE	SECOND DOSE	THIRD DOSE	FOURTH DOSE	FIFTH DOSE	SIXTH DOSE
Camphor monobromide	120	120	110*	90	70	50
Absinth	115	115	95*	55	40	

The asterisk marks the dosage at which the first true sustained clonic convulsion appeared. Following the appearance of convulsions, with periods of great muscular activity, the fall in blood pressure was much sharper in the post-convulsive period than after the injection of sub-minimal doses.

The respiratory rate is accelerated even after doses of absinth or camphor monobromide insufficient to elicit occasional twitches. The rate during the convulsion itself is obscured but following the convulsion it is sometimes slower than just previous to it. This slowing becomes more and more

TABLE 2

	BLOOD PRESSURE	HEART RATE	RESPIRATION
	mm. Hg		
First convulsion:			
Before	140	192	78
During	180	228	—
After	112	168	180
Second convulsion:			
Before	112	168	180
During	165	236	—
After	106	168	218
Third convulsion:			
Before	106	168	216
During	144	222	—
After	90	218	170
Fourth convulsion:			
Before	110	218	170
During	120	218	—
After	50	60	72

marked as the dose approaches the lethal amount. In the strong tonic phase following the lethal dose, the thorax may be compressed and held rigidly so that no air enters the lungs. The glottis is closed and there is a moaning sound as the air in the lungs is driven out. Subsequent to the general relaxation of the skeletal muscles, the respiration is slow and gasping and finally fails completely. Asphyxial movements of the limbs

may become apparent after the cessation of all clonic and tonic movements and even after the failure of the terminal gasps following the lethal dose.

A series of experiments was also done on intact animals in which the minimal convulsive dose was determined and repeated doses of camphor monobromide or absinth of the same strength were injected at suitable intervals, until the animal succumbed. In this way, the vaso-dilator effects of the drug were probably kept at a minimum and the cardio-vascular changes in successive convulsions were studied in more detail. Table 2 summarizes the heart rate, blood pressure and respiratory rate during four such convulsions.

It may be seen that while the convulsion is at its height, both heart rate and blood pressure are higher than at any other time. Occasionally two separate and successive convulsions followed only one dose of the convulsant agent. In some of these experiments, when the second clonic convulsion had persisted with severity for some time, blood pressure rose higher than it had in the first convulsion. At this time, the nose and the mucosa of the mouth were cyanotic. The record of the respiratory movements showed no true respiration of any degree of regularity, but only irregular movements which appeared to be due to the clonic contractions of the skeletal musculature. One got the impression from observing the sequence of events that the rise of blood pressure might be due in part to asphyxia as well as to the activity of the skeletal muscles (fig. 3).

There is sometimes also an increase in the latent period intervening between the injection of the convulsant agent and the appearance of the clonic twitches when the same dose is repeated at successive intervals. The following table, compiled from the results of the experiment of December 23, 1930, shows this.

TABLE 3

NUMBER OF INJECTION	DOSAGE—CAMPHOR MONOBROMIDE	LATENT PERIOD
	cc. per pound	*seconds*
One	0.023	12
Two	0.023	20
Three	0.023	21
Four	0.023	27

This may be a condition similar to the gradual increase of the latent period observed by one of us (C) between occlusion of the head arteries and the initiation of the anemic rise of blood pressure in successive short periods of anemia.

Following the final fall of blood pressure, the onset of rigor mortis has been observed in ten to fifteen minutes after the terminal respiratory gasps. This period may be shortened to five minutes (Pike and Notkin,

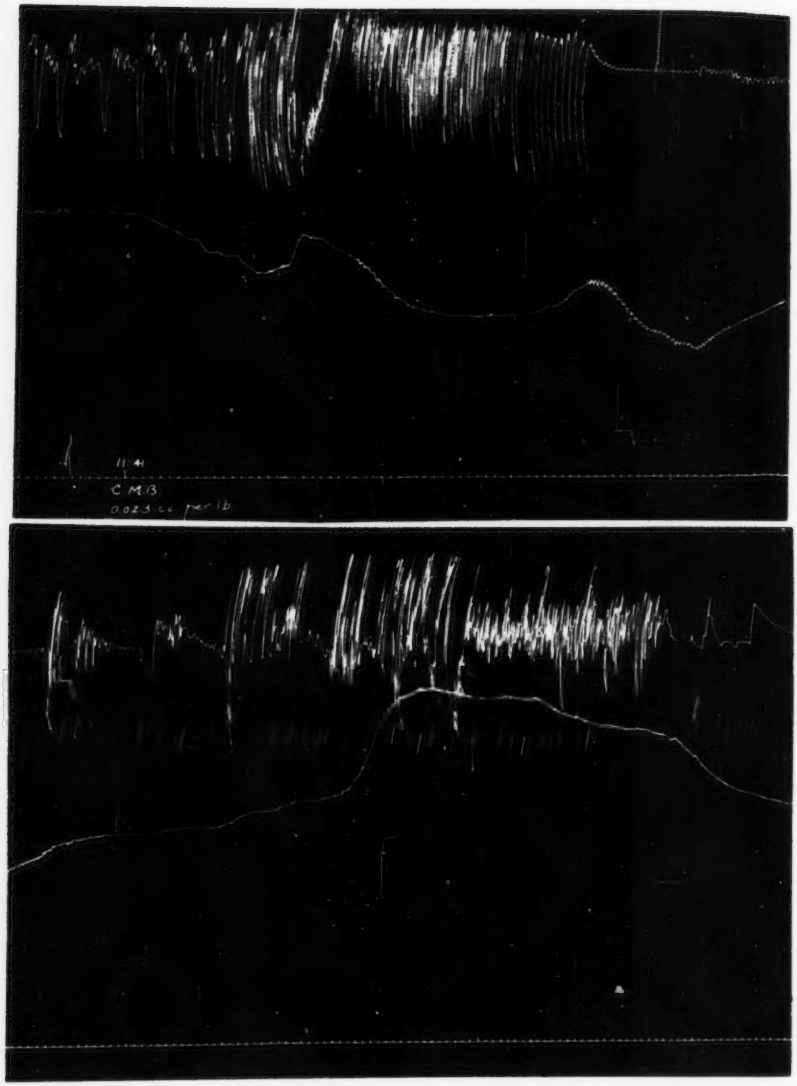

Fig. 3. Shows two successive convulsions following a single injection of camphor monobromide, on a rapid drum. The first convulsion, in the upper tracing, occurs during a fall in blood pressure. The second convulsion in the lower tracing, is accompanied by a rise of blood pressure most marked when the true respiratory movements were almost in abeyance, and the nose was cyanotic. Heart rate is most rapid when the blood pressure is at its greatest height.

loc. cit.) when caffeine is injected along with absinth. In general, the situation parallels that seen under intense and prolonged muscular activity.

Some incidental observations bearing upon the general problem may be cited here. Muskens (p. 4) cites Weiss' (1882) clinical description of the appearance of fits in the intervals between two successive groups of respiratory movements in Cheyne-Stokes respiration. In a control animal in our own series, in which bilateral vagotomy had been done under ether a few hours previous to the injection of absinth, the respiration was irregular with a cessation of the clonic movements due to the absinth just before a deep respiration and their resumption immediately afterward. No blood pressure was taken.

Muskens mentions also (p. 244) punctiform hemorrhages in the face and neck after an epileptic seizure. We have observed in practically all control animals in which the dose of absinth was made great enough to bring on severe convulsions, the appearance of petechial spots in the lungs when examined post mortem. The histological examination by Dr. T. E. Hughes shows ruptured alveoli and small hemorrhages.

C. The cardio-vascular response after injection of curare. The question arises whether the greater persistence of the systemic blood pressure in intact animals or in those with low transection of the spinal cord is due:

1, to the greater degree of central control over the peripheral vessels because of the greater number of efferent vaso-constrictor paths which are open, or

2. to the greater effect of the skeletal musculature in compressing the blood vessels during the convulsions.

If the persistence of the mean blood pressure is due to central control through the vaso-constrictor fibers, the mean arterial pressure should remain reasonably high when absinth or camphor monobromide is injected into a curarized animal without any lesion of the spinal cord. If, however, there is any considerable effect of the skeletal musculature in the maintenance of the mean arterial pressure during a convulsion, the pressure should fall markedly on the injection of the drugs in a curarized animal.

The experimental results show a great fall of blood pressure on injection of either drug in a curarized animal.

December 9, 1930. Female cat, weight 7¼ pounds.

11:20 a.m. Novocaine in both Scarpa's triangles. Blood pressure 120 mm. mercury. This level was maintained until 11:52.

11:52 Curare injected intravenously, artificial respiration maintained through catheter in trachea. Blood pressure falls to 60 mm. mercury.

12:20 p.m. Absinth, 0.013 cc. per pound; total 0.10 cc. blood pressure fell to base line. Adrenalin administered—2 cc. of 1:50,000—and pressure rose.

1:05 Absinth, 0.20 cc. per pound; total 0.15 cc. blood pressure fell to base line, with no cardiac impulse showing.

Post-mortem. Heart muscle flabby, thymus small, stomach only slightly dilated.

The general rise of arterial pressure is present in curarized animals during cerebral anemia but is considerably reduced in extent.

In etherized animals, without lesion of the spinal cord, injection of moderate doses of camphor monobromide was followed by a more marked fall of blood pressure than occurred in unanesthetized control animals after doses of corresponding magnitude. In the etherized animals, there was a spontaneous recovery of the systemic blood pressure.

Discussion. Some comments may be made on the results of these experiments. They show that the maintenance of the systemic blood pressure, or its rise during a convulsion of experimental origin (absinth) is due largely to the skeletal musculature. This is similar to an earlier observation that the first asphyxial rise of blood pressure in the spinal animal disappears under curare (Stewart, et al., 1906, p. 300; Pike, 1913, p. 17). The main vascular action of the two drugs used is dilatation. The fact that a vaso-dilator drug may elicit convulsions comparable to true epileptic seizures lends no support to the view that the onset of an epileptic seizure is due to cerebral vascular spasm. It is possible, although unlikely, that these drugs may exert a constrictor action upon the cerebral vessels. A decreased blood flow through the brain in unit time, as well as a decrease in caliber of the cerebral arteries, would result directly from a fall in the systemic blood pressure. The situation in the brain is very similar to that described by Bowditch and Warren (1886), Pike, 1913, p. 21, in the limb of an animal to which the nerve supply has been cut, on the injection of curare. The limbs to which the nerve supply is intact, show an increase in volume, while the denervated limb shows a shrinkage.

The best observations available show that the blood pressure may rise or may fall during an epileptic seizure (Notkin) in patients without discoverable cardio-vascular lesions. There would seem to be no constant vaso-constrictor element in epilepsy and we incline to the belief that the changes in blood pressure during a convulsion are due largely to the action of the skeletal muscles.

The great fall of blood pressure seems the most probable cause of death from absinth. The course of events in a convulsion of experimental origin appears to be the contraction of the thoracic muscles with the forcible expulsion of the air from the lungs and the conseqquent reduction of the amount of oxygen available to the tissues. At the time of the relaxation of the general tonic spasm which, in our experience, immediately precedes death, the vaso-dilatation is so marked that a great fall of blood pressure would immediately follow the cessation of the adjuvant action of the skeletal muscles in maintaining blood pressure. We have repeatedly found the persistence of the heart beat on opening the thorax after the last respiratory movement has occurred, in one case, as long as thirty-two minutes afterward. Death does not appear to be primarily at least,

cardiac death, but attempts to revive the animal at this juncture have been unsuccessful. We do not know why.

The general picture is somewhat similar to that observed after high transection of the spinal cord early in the recovery from temporary ligation of the head arteries. Unless the blood pressure is raised artificially, the animals invariably die within a few minutes. The damaged medulla oblongata because of interruption of the vaso-constrictor pathway, cannot get back the blood pressure to facilitate or even to maintain, its recovery (Stewart et al., 1906; Pike and Coombs, 1917). Yates (1921) observed sudden collapse of cats following severe muscular effort of the fore limbs and head, even when the animal was on its back, in the first few days after high transection of the spinal cord. The ordinary remedial measures failed to effect recovery. The same course of events was observed by Bowen, Pike and Coombs (1922). This suggests that some product of strong muscular activity liberated in the circulation, may cause vaso-dilation in regions of the animal over which the bulbar mechanism has not yet acquired sufficient vascular control. We have never seen this condition in animals which have survived high spinal transection for five to ten days or more. One might expect that the liberation of some vaso-dilator substance by the skeletal muscles and the vaso-dilator effect of the camphor would be summed up to produce a greater vaso-dilatation than would result from either one alone. The successive decreases in blood pressure shown in table 1 may be related to such an action.

It is possible that the condition of the peripheral vessels induced by absinth or camphor monobromide is such as to prevent any effective vaso-motor action upon the blood vessels. The injection of adrenalin or ephedrine at the time of the failure of blood pressure is frequently followed by a return of respiratory movements. Since adrenalin has but brief action, the greater persistence of the convulsant drugs in the circulation may again bring about a fall of blood pressure from causes which probably do not lie primarily or at least, wholly, in the central nervous system. Ephedrine, because of the greater duration of its effect, is more efficient in restoring the animal than adrenalin.

After adrenalectomy the generally weakened action of sympathetic nerve on smooth muscle may render the peripheral vascular system more vulnerable to the vaso-dilator action of the convulsant agents. This suggests a possible explanation for the greatly decreased lethal dose after adrenalectomy, but the reason for the decreased minimal convulsive dose is not yet clear. The dilatation of the peripheral vessels after the administration of absinth or camphor monobromide is also shown by the observation of Hill and Nabarro (1895) that "The blood flow from the deep femoral vein of the dog during an epileptic fit (excited by essential oil of absinthe) is three to five times as great as during rest." Hill (1910) states that the

flow from the torcular Herophili is also increased. This is in accord with the observations on the increased flow of blood to active glands or muscles generally. One would expect a similar peripheral vaso-dilatation during the intense muscular activity of an epileptic seizure which, if uncompensated for, would lead to a fall of systemic blood pressure. In the absence of any known direct action upon the central vasomotor mechanism in essential epilepsy, the rise of blood pressure observed clinically during a convulsion is due largely to the activity of the skeletal muscles.

The fall of blood pressure through peripheral vaso-dilatation would, on the basis of Marey's law (Erlanger and Hooker, 1904), lead one to expect the most marked acceleration of pulse rate at the period of the lowest blood pressure during the convulsions. Wickwire (1920) found that this compensatory change in heart rate with changing blood pressure fails in the deeply anesthetized animal. The changes in heart rate and blood pressure during convulsions, whether observed clinically or experimentally, should give us some index of possible derangements of the nervous regulation of the cardio-vascular system. The facts (table 2) indicate a departure from Marey's law under certain experimental conditions.

Since the acceleration of the respiratory rhythm after absinth or camphor monobromide seems much greater than could be accounted for by the mere change in volume of blood flowing through the medulla, (Hastings, Coombs and Pike, 1921) some direct action of these drugs upon the central respiratory mechanism is indicated. The mechanism of this action has never been analysed in the light of the newer conceptions of the functional organization of the nervous mechanism for respiration (Coombs and Pike, 1930). This direct action may participate as a cause of death, although the respiratory movements persist after all clonic or tonic movements of the skeletal muscles have ceased. There is the same group of terminal respiratory gasps in death from these convulsant agents as occurs in death from almost any cause which does not involve direct anatomical injury to the central respiratory mechanism itself. The injection of adrenalin or ephedrine will often restore respiration which has given every evidence of impending failure if cardio-vascular failure has not progressed too far.

The high pulse rate observed at the height of the rise of blood pressure indicates some more or less direct action, either of the convulsant drugs or of some condition set up by them, upon the nervous mechanism of the heart.

We should say here that we do not regard the method of inducing convulsions by the action of any drug as the direct counterpart of essential epilepsy as it is observed clinically. We are merely attempting to duplicate by experimental means some of the conditions observed clinically. The greater the number of these conditions which can be reproduced experimentally, the more nearly will the experimental analysis be complete

GENERAL SUMMARY OF RESULTS

1. Immediately following intravenous injection of absinth or camphor monobromide there is a fall of blood pressure. The degree of this fall bears a general relation *a*, to the amount of the drug injected; *b*, to the degree to which the reactions of the skeletal muscles are eliminated; *c*, to the degree of anatomical injury to the efferent vaso-motor pathways, and *d*, to the degree of anesthesia.

a. The effect upon blood pressure of a small (sub-convulsive) dose of absinth or camphor monobromide is a slight fall of blood pressure, recovery from which generally occurs spontaneously, under all the experimental conditions which we have described in this paper. Successively larger doses lead to increasingly severe depressions of blood pressure from which recovery may not occur spontaneously.

b. When the spinal cord is transected as high as the level of the third thoracic segment, the blood pressure falls on the injection of either drug and does not rise during the clonic activity of the skeletal muscles, or may even continue to fall during a part of the time a convulsion is in progress. A clonic convulsion may occur with a falling blood pressure. Sometimes no convulsion occurs before the lethal dose is reached.

When the transection of the spinal cord occurs at the level of the seventh segment or lower, the preliminary fall of blood pressure consequent to the injection of the drug is arrested at the time the clonic convulsions appear and thereafter becomes a rise.

When the spinal cord is intact, the same conditions obtain as after a low transection, but the rise of blood pressure may be even greater during the period of the clonic convulsions.

When the skeletal muscles are eliminated from participation completely by the intravenous injection of curare, the blood pressure falls on the injection of either drug, a small dose in this case being the lethal dose.

c. The degree of injury to the efferent vaso-motor pathways on transection of the spinal cord runs more or less parallel to the degree of injury to the efferent motor pathways to the skeletal muscles. The effects of curare indicate that the skeletal muscles are more important in the production of the rise of systemic blood pressure during the period of a clonic convulsion than the vaso-motors.

d. Under moderate anesthesia, the blood pressure falls, but recovery is spontaneous without the appearance of any clonic convulsions, although it takes place much more slowly than when a convulsion occurs.

2. In conditions of low systemic blood pressure, it is difficult to elicit clonic convulsions; but they immediately appear at the same dosage if the blood pressure is raised by the injection of absinth or camphor monobromide, or by mechanical compression of the descending aorta. A vascular spasm, so far as can be seen from the evidence now at hand, does not appear to be a necessary condition in the genesis of a convulsion.

3. Respiration is accelerated at a lower degree of dosage of absinth or camphor monobromide than the minimal convulsive dose; at a high dosage, respiratory failure and failure of blood pressure occur simultaneously although the heart may continue to beat for some minutes after the terminal respiratory gasps.

4. The convulsions arise from the actual excitation of the motor cells of the cerebrum by absinth or camphor monobromide. The excitability of the cells may be affected by various conditions, such as blood supply, oxygen, carbon dioxide and anesthetics. Work must be done to stimulate a nerve cell and the total amount of work necessary may be divided among several agents. Differently stated, there is a summation of stimuli of different kinds in the genesis of the convulsion.

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A METHOD FOR UNIFORM STIMULATION OF THE SALIVARY GLANDS IN THE UNANESTHETIZED DOG BY EXPOSURE TO A WARM ENVIRONMENT, WITH SOME OBSERVATIONS ON THE QUANTITATIVE CHANGES IN SALIVARY FLOW DURING DEHYDRATION

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The effect of two or three days' water deprivation in reducing the quantity of fluid secreted by the salivary glands has been reported by several observers. Pavlov (1902), without giving methods or numerical results, states that "the quantity of the digestive juices in general stands in close connection with the content of water in the organism." Cannon (1918) measured changes in salivary flow on himself over a period of some twenty hours during which he drank no water, but in spite of efforts at uniform stimulation and the exclusion of psychic effects the salivary flow was not constant. More recently Crisler (1928) has demonstrated that the conditioned salivary secretion induced in a dog by morphine can be practically abolished by five days' dehydration, and that the unconditioned secretion is also reduced, although not so much. Gantt (1929) found that the salivary secretion obtained from feeding bread crumbs, salt herring and ham to two children was diminished by one-half after twelve hours' thirst; forced water intake did not increase the secretion markedly above normal. Winsor (1930) followed quantitatively the parotid secretion of a man for 70 hours of thirsting and obtained results similar to Cannon's, both in reduced salivary flow and its fluctuations.

Rapid loss of water from the body also is attended by marked decrease in salivary secretion. Cannon (1918) found that a loss of 500 cc. by sweating reduced his salivary flow by one-half, and Aggazzotti (1922) observed during mountain climbing a lessened salivary secretion related to the loss of water and salts by sweating.

In order to study the physiological mechanism which reduces the salivary flow during dehydration, a method for uniform stimulation of the salivary glands is needed that permits a comparison of results obtained over a considerable period of time. The purpose of this paper is to describe such a method which, without introducing abnormal conditions, gives reliable information on the salivary flow in the unanesthetized dog.

METHOD. In dogs (devoid of sweat glands) exposure to heat produces respirations as rapid as 200 to 350 per minute. The mechanism is reflex and mediated chiefly by cutaneous sensory nerves (Sihler, 1879). The efficiency of this cooling mechanism is often so great as to overcompensate, so that a gradual but definite fall in the body temperature occurs (Welikanoff, 1928). If immediate panting is prevented, e.g., by light anesthesia, polypnea will still appear, due to a central nervous discharge, when the body temperature reaches 41.7°C. (Richet, 1898). Lucksinger (1877) quite incidentally observed that overheating and panting in dogs is attended by an extra flow of saliva. Excessive drying of the mouth and tongue is prevented by greater activity of the salivary glands. Welikan-

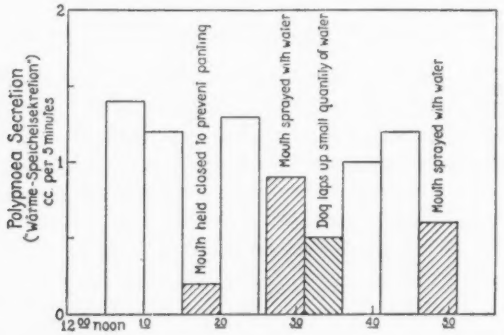

Fig. 1. Experiment in warm-room at 40°C. "Mrs. Whity" ♀ dog 10 kgm. Double submaxillary fistulae, left vago-sympathetic cut. Saliva collected from right submaxillary fistula. The dog panted continuously at about 300 per minute except for the five minutes during which its mouth was held closed.

It may be seen that the "Wärme-Speichelsekretion" almost stopped while panting was prevented, and was markedly reduced after spraying the mouth with a few cubic centimeters of water. In both cases the tendency for the buccal mucosa to become dry was diminished.

off made some observations (unpublished, but reported by Babkin, 1928) on this so-called "Wärme-Speichelsekretion" in dogs with fistulae of the submaxillary and parotid glands. It is evident from his work that the panting and secretion rates go hand in hand as the dog is exposed to higher and higher external temperatures, or placed in the direct rays of the sun, but it cannot be definitely concluded that the secretion is a result of the panting rather than an attendant phenomenon, perhaps brought about by direct influence upon the salivary glands of the nervous centers that determine the panting. Zebrowski's observation (1905) that a flow of saliva is called forth by merely holding the mouth open and exposing the mucous membrane to the drying action of the air, leads one to suppose that it is

the dryness due to the panting that stimulates the salivary glands. The importance of this reflex in keeping the mouth continuously moistened has been emphasized by Cannon (1918).

That the "Wärme-Speichelsekretion" follows a drying of the mouth and is not due directly to the nervous center that controls panting, is indicated by two simple experiments. If secretion and panting resulted from the same central discharge, interference with the panting by mechanical closure of the dog's mouth should not reduce the secretion. Nevertheless, this does occur (see fig. 1). If, on the other hand, the "Wärme-Speichelsekretion" is due to dryness of the mouth, the stimulus for secretion should be reduced without interfering with the panting by simply moistening the tongue. Indeed, Welikanoff reports that the "Wärme-Speichelsekretion" is immediately lessened by swabbing the tongue with a wad of wet cotton, or allowing the dog to drink a small quantity of water (50 cc.), but he fails to state whether or not there was a corresponding fall in the panting rate. In figure 1 may be seen some of my results bearing upon this question. In the first place, mechanically closing the mouth practically abolished the secretion. In the second place, squirting 2 cc. of water over the tongue every few minutes reduced the salivary flow one-half without changing the panting rate except for a moment when the dog swallowed the water. The "Wärme-Speichelsekretion," therefore, is a reflex due to the drying of the mouth produced by panting, and not due directly to the stimulus of the heat, or to a rise in body temperature for, as a rule, this remains constant. In view of these facts, I propose "polypnea secretion" in place of "Wärme-Speichelsekretion" to indicate its dependence upon the panting.

A constant rate of panting can be produced in an atmosphere of constant temperature, and with a fairly uniform humidity the evaporation from the mouth then remains unchanged. This method for uniform stimulation of the salivary glands makes use of the normal reflex mechanism for secretion. The panting rate may be considered an index to the intensity of stimulation. The dependability of the method is shown by the consistent results obtained.

EXPERIMENTS AND RESULTS. In one of the six dogs used I made a single submaxillary fistula, but in the remaining five I established double fistulae. In moderately small dogs with double submaxillary fistulae the openings of the ducts are close together, but by using small salivary funnels, it is possible to collect from both ducts separately. The salivary funnels were attached by means of Mendelejeff's cement. Saliva was collected in 5 cc. glass tubes, graduated in 0.1 cc., but these were not hung directly from the salivary funnels as described by Pavlov; instead, I found it better to suspend them by a small rubber band passed over the dog's head. This was necessary in the warm-room where the cement tends to soften and the funnel is easily dislodged.

The experiments were performed in a warm-room, in which a number of preliminary trials were made at various temperatures ranging from 35° to 49°C. At the lower temperatures panting was less prompt in its appearance and was irregular. The salivary flow reflected the fluctuations. On the other hand, at 49°C. panting was very rapid; rates of over 400 per

TABLE 1

Observations on the polypnea secretion in the same dog; a, after being deprived of water and b, after having had plenty of water*

TIME	PANTING	FLOW OF SALIVA FROM SUBMAXILLARY FISTULAE	
		Left	Right
a. October 13, 1930. No water for 48 hours			
	per minute	*cc. per 5 minutes*	*cc. per 5 minutes*
10:40 a.m. Enter (Room temperature = 40°C.)			
10:54	240		
10:54–10:59		1.7	1.3
10:59–11:04	300	1.6	1.5
11:05–11:10	300	1.6	1.3
11:10–11:15	300	1.5	1.4
11:15–11:20	300	1.5	1.5
11:21–11:26	300	1.7	1.4
11:26–11:31	310	1.6	1.5
11:31–11:36	310	1.8	1.5
11:37–11:42	320	1.5	1.6
11:42–11:47		1.5	1.4
		1.6	1.4 Av.
b. October 15, 1930. Normal water intake for 24 hours			
11:45 a.m. Enter (Room temperature = 40.2°C.)			
12:08 p.m.	300		
12:13–12:17	300	3.0	3.2
12:18–12:23	300	3.1	3.2
12:23–12:27	300	3.1	3.4
12:30–12:35	300	3.0	3.2
12:48–12:53	300	2.9	3.0
12:53–12:56	300		3.3
		3.0	3.2 Av.

* Mike, ♂ dog, 13.4 kgm. Double submaxillary fistulae established August 15, 1930. Left vago-sympathetic cut October 8, 1930.

minute were common, and although the saliva poured forth in much greater abundance than at lower temperatures, the secretion rates were less steady; after 10 to 15 minutes the body temperature rose and the dog showed signs of distress. Moreover, the Mendelejeff's cement became so soft that the salivary funnels soon fell off.

TABLE 2

Daily observations on the polypnea secretion

DATE	TIME	ROOM TEMPERATURE	PANTING	POLYPNEA SECRETION (SUBMAXILLARY FISTULAE)	
				Left	Right
"Mike," ♂ dog, 13.4 kgm. Double submaxillary fistulae established August 15, 1930					
		°C.	*per minute*	*cc. per 5 minutes*	*cc. per 5 minutes*
October 7	9:30 a.m.	39.7	300	3.8	3.5
October 8	11:00 a.m.	39.9	300	3.1	2.9
October 8	p.m.	Left vago-sympathetic cut. Ether anesthesia*			
October 9	11:30 a.m.	40.0	300	3.2	3.3
October 10	11:30 a.m.	39.9	280–300	3.0	2.8
October 11	12:00 noon	40.2	300	3.1	3.1
		No water for 72 hours			
October 13	11:30 a.m.	40.0	310	1.6	1.4
October 14	10:30 a.m.	40.0	300	1.5	1.5
October 14	12:00 noon	Drank 1200 cc. water			
October 14	3:00 p.m.	40.2	290	2.2	2.3
		Water *ad libitum*			
October 15	12:30 p.m.	40.2	300	3.0	3.2
October 21	12:00 noon	39.6	300	2.9	3.2
"Czermak," ♂ dog, 10 kgm. Double submaxillary fistulae established August 15, 1930					
October 7	11:00 a.m.	39.7	300	2.4	3.1
October 8	10:00 a.m.	40.0	270	2.0	2.8
October 8	p.m.	Left vago-sympathetic cut. Ether anesthesia*			
October 9	12:00 noon	40.0	280	1.9	2.8
October 10	12:30 p.m.	40.0	260–70	2.0	2.7
October 11	12:00 noon	40.2	280	2.0	2.8
		No water for 72 hours			
October 13	11:30 a.m.	41.0	280	0.95	1.3
October 14	10:30 a.m.	40.0	270	0.4	0.5
October 14	12:00 noon	Drank 1000 cc. water			
October 14	3:00 p.m.	40.2	300	0.9	1.3
		Water *ad libitum*			
October 15	12:00 noon	40.2	300	1.4	2.0

* The question of the possible relation of the cervical sympathetic to the control of salivary secretion during dehydration will be considered in a later communication. So far, the evidence has been conflicting.

In the end, 40°C. was chosen as the most satisfactory temperature. Panting appeared within 2 or 3 minutes, except when the dog was very

thirsty, and remained for an hour at about 300 per minute without noticeable variation. Figures for panting rates should be regarded as only approximate. The practice was to count to the nearest 10 the respirations occurring in 20 seconds; repeated countings have indicated that the error is about 5 per cent. Rectal temperatures did not change; and the polypnea secretion was reasonably constant, as demonstrated by the results reported in table 1. Both tests given in that table as illustrations were made on the same dog, "Mike," who is very quiet by nature and seldom moves around when we are together in the warm-room. The results in table 1 (a) were obtained after the dog had been deprived of water for 48

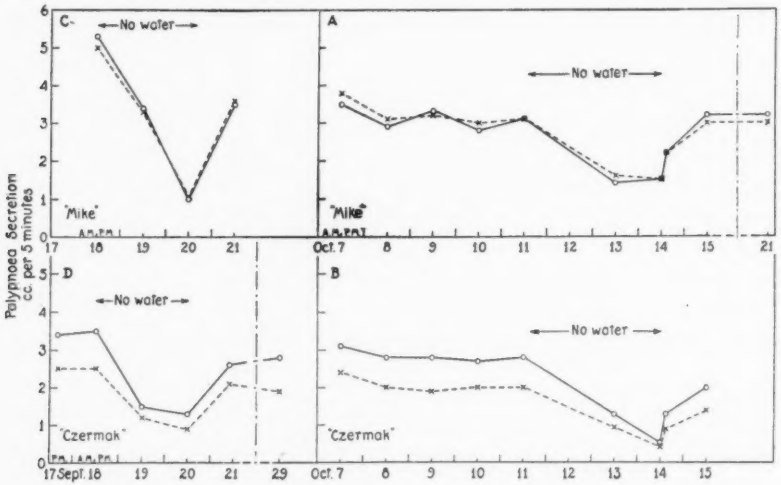

Fig. 2. Curves showing the effect of changes in water intake upon the polypnea secretion. Saliva collected from right (0——) and left (x——) submaxillary fistulae in dogs panting at about 300 per minute.

The left vago-sympathetic was cut in both dogs on October 8 after the determinations of polypnea secretion had been made for that day.

hours; those in table 1 (b), after he had been given plenty of water for 24 hours.

The results show how remarkably uniform the polypnea secretion can be maintained. When a dog is taken into the warm-room the secretion soon reaches a level where it stays as long as the dog is exposed to these conditions. In one case, a dog was kept in the warm-room for more than two and a half hours, and at the end the salivary flow was the same as at the beginning.

In the manner described above, the polypnea secretion was determined

from day to day in dogs given water *ad libitum* or deprived of water. Some of the results obtained on the dogs "Mike" and "Czermak" are set forth in table 2; the room temperature and the average rate of panting during each determination are also included. In order to avoid any effects from the afternoon feeding, the tests were always made by the middle of the day. It may be seen that for five successive days during which the water intake was normal, there were only small fluctuations in the polypnea secretion (see also fig. 2, A and B).

Immediately after this five-day control period, "Czermak" and "Mike" were deprived of water for three days. The effect of the dehydration upon the polypnea secretion is evident, and the parallel behavior of the two submaxillary glands in each dog makes the results more striking and

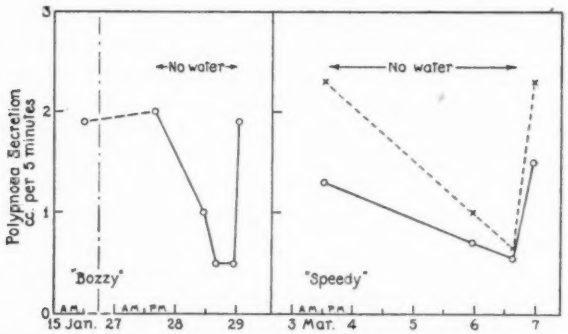

Fig. 3. The effect of water deprivation upon the polypnea secretion, showing especially the promptness with which the secretion may return to its normal rate when water is given to the dehydrated dog. Saliva collected from submaxillary fistulae (0—— right, x——left).

indicates not a haphazard response, but an exact mechanism sensitive to changes in the water content of the body. As seen in the graphs, the fall in salivary secretion was relatively greater in "Czermak" than in "Mike" and, indeed, the former showed signs of distress that were not apparent in the latter. Likewise, "Czermak's" recovery from the dehydration was more sluggish, judging from the salivary flow; even after twenty-four hours of free access to water, the polypnea secretion had not returned to its original level, while in "Mike" the recovery seems to have been complete by this time.

Four other typical experiments showing the effect of dehydration upon the polypnea secretion are presented graphically. Since two of these were done on "Mike" and "Czermak," they have been included in figure 2, C and D, preceding A and B in chronological order. Figure 2, therefore,

shows all the observations made on the two dogs for a period of about a month, during which time they were deprived of water twice, first for two days and later for three days. The other two experiments (see fig. 3) were the first attempted, and were not carried out under quite the same conditions, nor with the same precautions observed later. The salivary tests were made at no particular time of the day, although they were never made immediately after feeding the dogs. The temperature of the warm-room was 35° to 36°C. for the first experiment (fig. 3A) and varied between 39° and 42°C. for the second (fig. 3B). Panting rates were not determined; the dog was simply kept in the warm-room for half or three-quarters of an hour until the polypnea secretion became fairly constant.

The promptness with which the polypnea secretion may return to normal when the animal is given water after a period of dehydration is well shown in figure 3. "Speedy," who was without water for 74 hours, showed a normal polypnea secretion when a test was made 6 hours after giving water again, and "Bozzy," who had had no water for 32 hours, with the result that the polypnea secretion had fallen from 2 cc. per 5 minutes to 0.5 cc. per 5 minutes, again secreted at the rate of 1.9 cc. per 5 minutes when taken into the warm-room only 2 hours after drinking 1 liter of water. The recovery may be more rapid than these experiments demonstrate.

In all, nine experiments have been performed on six different dogs to show the effect of dehydration on the polypnea secretion, and although the extent to which the salivary flow falls as the result of two or three days' dehydration differs a good deal, not only in different animals but in the same animal from time to time, the general character of the effect is the same in every case. Of course, variations are to be expected as a result of differences in water content of the body at the time of the experiments, or differences in rate at which water is lost from the body during the period of water deprivation.

Other observations were made in the course of these experiments which deserve to be mentioned.

The reluctance with which a dehydrated dog begins to pant is in striking contrast to the ease and promptness with which panting appears when the animal has had plenty of water. Usually, panting commences within 2 to 3 minutes after we enter the warm-room; it begins at about 200 per minute but in 10 minutes has reached a fairly constant maximum of 300 per minute; once started, it is continuous and its appearance is not attended by any evidence of restlessness. But after two or three days without water, the dog may sit very still for 15 to 20 minutes after it is brought into the warm-room, breathing very slowly and quietly; and when panting does appear, it comes first in short spells accompanied by considerable restlessness. The dog may bark or make some attempt to get away, but after 5 or 10 minutes it again becomes quiet and pants continuously at the usual

rate. The saliva is scant and sticky; mucus collects on the tongue; repeated attempts are made to swallow.

After such tests as the one just described, I have made it a practice to give the dog a few laps of cold water, even when the water deprivation was to be continued. This was done not only to relieve the discomfort but also to keep the mouth as clean as possible.

Gradual changes occurred in the salivary flow, which were probably not related to fluctuations in the water content of the body. In the course of the month during which observations were made more or less constantly on "Czermak" and "Mike," there was some tendency for the rate of polypnea secretion to decrease. In the warm-room at 40°C. "Mike," for instance, at first secreted at the rate of 5 cc. in 5 minutes, but after more than a month of experimenting with him this had fallen to 3 cc. in 5 minutes. The diagrams in figure 2 make this clear. The same general decrease is shown by the records of "Czermak."

I have seen a gradual and extreme reduction of salivary secretion in a much more striking case. "Hector," a large male police dog, was chosen for the establishment of double submaxillary fistulae because he had a particularly wet mouth. About three weeks after the operation, the response of the submaxillary glands to all sorts of stimuli, such as feeding the dog cookies or giving him lemon juice, began to show marked diminution. A few days later even lemon juice failed to call forth more than a few drops of saliva from the submaxillary fistulae. That this was a general phenomenon of salivary secretion and not confined to the submaxillary glands, was indicated by dryness of the mouth and absence of the glistening appearance characteristic of the normal dog. In all other respects the dog seemed to be in good health. The diet was changed to include a certain amount of raw meat, but no improvement was seen in the salivary secretion.

Discussion of the method. Inasmuch as the salivary glands are inactive unless stimulated (Mitscherlich, 1832), the effect upon the salivary flow of changes in physiological conditions in the body can only be measured as far as they affect the secretion during application of a uniform stimulus. For this reason it is of importance to have a convenient method for uniform stimulation which may be combined with experimental procedures of various sorts. The polypnea secretion represents such a method, one which requires no anesthesia, one which involves an entirely normal reflex for secretion, one which can be applied constantly for hours with remarkable regularity in the response, and which may be conveniently combined with experimental procedures designed to affect the activity of the glands (hemorrhage, intravenous injection, etc.). Moreover, psychic effects are largely eliminated and the panting is a fair index of the intensity of stimulation.

It must be pointed out, however, that since dryness of the mouth is the

immediate cause of the polypnea secretion, the intensity of the stimulus produced by a given rate of panting is somewhat greater in the dehydrated dog where salivary flow is scant than in the normal dog where it is abundant. Correction for this error would only serve to exaggerate the effect of dehydration upon the polypnea secretion.

SUMMARY

Uniform stimulation of the salivary glands in the unanesthetized dog may be obtained by exposing the animal to a warm atmosphere of constant temperature, thereby producing always the same rate of panting (polypnea). As panting dries the mouth and tongue, the salivary glands are reflexly stimulated to secrete a thin, watery saliva which may appropriately be called the "polypnea secretion."

Holding the dog's mouth closed or spraying the mouth with water promptly lessens the polypnea secretion (cf. fig. 1).

In a warm room with a temperature of 40°C. the polypnea secretion reaches a definite level within 10 to 15 minutes and has been observed to stay constant for over two hours (cf. table 1).

On a normal water intake the polypnea secretion remains nearly the same from day to day (cf. table 2), but after 24 hours without water it is often reduced to one-half the normal rate, and after 3 days' water deprivation it may fall to one-fifth normal (cf. table 2 and figs. 2 and 3).

This method of uniform stimulation of the salivary glands is being used in an attempt to uncover the physiological mechanism responsible for the reduction in salivary flow that accompanies dehydration.

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THE RELATIVE INCREASE IN METABOLISM OF THE LIVER AND OF OTHER TISSUES DURING PROTEIN METABOLISM IN THE RAT

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It has been assumed that the various organs and tissues of which the body is composed participate more or less uniformly in the increased heat production which follows protein ingestion. Aub suggested (1, 2) that the effect might be "located in the viscera," but came to no conclusions on this subject. Even those who believe that the specific increase in metabolism represents the work required for the intermediary metabolism of the amino-acids have not attempted to show that certain tissues participate in this metabolism to a greater extent than others. Wilhelmj and Mann (3), (4), (5) demonstrated that deaminization and the specific dynamic action of amino-acids were both abolished by hepatectomy. They considered that intermediate products of metabolism, rather than amino-acids, were the stimulators of cellular oxidation (6) throughout the body.

In anesthetized rats it is possible to make rapid observations of the rate of oxygen absorption before and after excluding various tissues from the circulation by ligating the blood supply. It is obvious that this method can be used to study oxygen requirement of different organs in animals on high and low protein diets in order to determine the intensity of metabolism in various tissues.

METHODS. Male white rats of various ages and weights were used in all experiments. Equal numbers of litter-mates were put on the two diets for 3 to 5 days before the determinations were made in order to have the same number of observations under identical environmental conditions for each group. The high protein group received 74 per cent casein, the low protein group 74 per cent cornstarch; both received 10 per cent each of cod liver oil and yeast extract, 4 per cent of salt mixture and 2 per cent alfalfa. There was no period of starvation.

To determine the oxygen consumption, a tracheal cannula was used in connection with a spirometer made from a 30 cc. luer syringe with a light bakelite "float" closely fitting and sealed with kerosene. Carbon dioxide absorption was effected by soda lime in a glass tube 6 cm. long which connected the tracheal cannula with a stoppered bottle into which the

spirometer, thermometer and tube from the oxygen tank also led. This bottle was nearly filled with strongly alkaline solution, the tubes from the spirometer and tracheal cannula ending a few millimeters above the surface of the fluid. The temperature of this system did not vary by more than 0.2°C. during the time required for 4 or 5 successive determinations of absorption rate. The rate was measured by the time required for the "float," which oscillated with respiration, to fall 2 cc. This was corrected for temperature and barometric pressure and for the calibration of the syringe. The rats were anesthetized by the intraperitoneal injection of 16 mgm. chloretone in 0.2 cc. 95 per cent alcohol for each 100 grams of rat. The body temperature, 35 to 36.5°C., varied with the environmental temperature and fell 0.5 to 0.7° during the entire period of observation, including opening the abdomen. The fall of O_2 metabolism during laparotomy and placing the ligatures averaged 4 per cent.

In order to determine the metabolism of various organs ligatures were placed loosely about the blood vessels, the rate of O_2 absorption noted for 3 to 6 volumes of 2 cc. each, and the ligature then tied. After the subsequent rate of absorption had become constant for 3 to 5 periods, the abdomen was opened and the completeness and position of the ligature established by cutting the vessels distal to it. The renal arteries and veins were ligated in one group of rats; the abdominal aorta below the renals was ligated in another, and in half of these (equally divided between rats fed high and low protein diets) the inferior vena cava was included in the ligature. The ligature for the splanchnic vessels passed between the esophagus and aorta above and the descending colon and aorta below, and included the entire arterial supply of the stomach, gut, spleen, pancreas and liver. The collateral circulation, in experiments of such short duration, was probably very small even in the liver. All results were recorded in terms of O_2 per minute per 100 sq. cm. body surface. The formula 11.34 $\times$ body weight to the $\frac{2}{3}$ power was used although more recent work indicates that a much smaller constant, perhaps as low as 7.47, is more accurate (7).

Results. As had been noted in previous (unpublished) experiments in this laboratory, carried out on rats on different diets, the specific dynamic action of protein was apparent at room temperature (18°–26°C.) when the animals were anesthetized with chloretone-alcohol. The metabolic level was slightly higher with higher environmental temperature, because of the slower drop in body temperature. In unanesthetized rats the metabolism rises when the environmental temperature falls below 28°C. The effect of environmental temperature was minimized in this experiment since the conditions were the same for rats on high and low protein diets.

The results of the experiments are summarized in figures 1 and 2. The renal vessels were ligated in only three rats on each diet, since the results

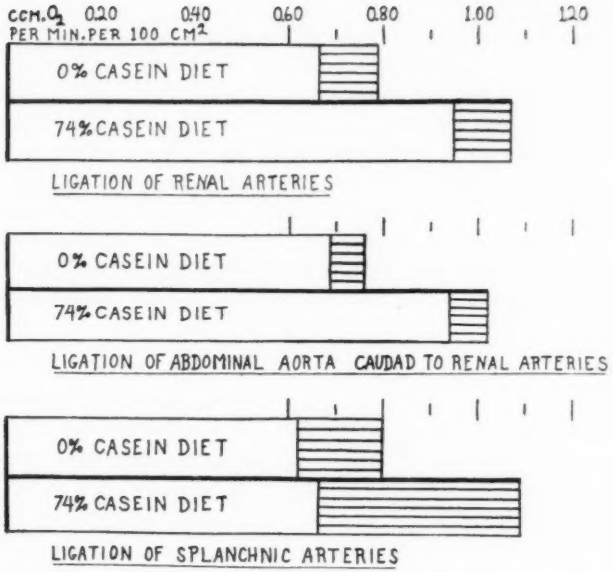

Fig. 1. The rates of oxygen consumption of rats, on high and low protein diets, before and after ligating the blood supply of various regions. The total length of the columns indicates the oxygen consumption before, and the unshaded portion the rate after ligation. Note the great decrease in O_2 consumption of the rats on 74 per cent casein diet when the abdominal viscera are deprived of blood supply.

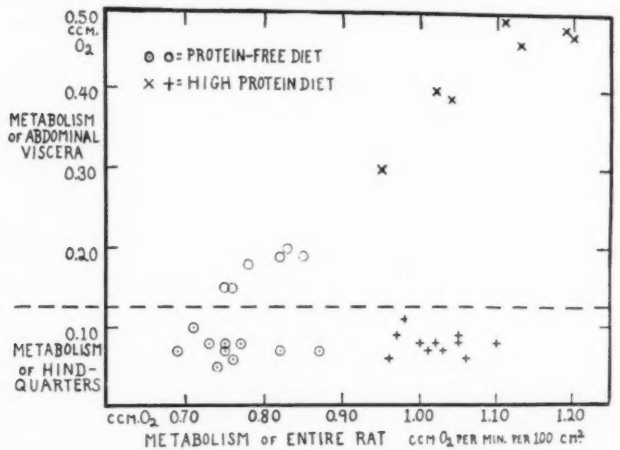

Fig. 2. The rate of oxygen consumption of individual rats, before ligation of blood supply of certain organs, is plotted against the O_2 requirement of the region rendered ischemic by ligature. The O_2 consumption of the hind-quarters is about the same in the rats on high and low protein diets, while the abdominal viscera of the former group need, on the average, 2.4 times as much O_2 per 100 cm. per minute as the rats on a diet nearly protein-free.

were so nearly the same in both groups and it was not certain that urine secretion was normal under these conditions. There was no evidence of a specific dynamic action upon renal metabolism, although the metabolism of the rats on 74 per cent casein was elevated 35 per cent.

In the groups of rats (13 animals) in which the splanchnic vessels were tied the O_2 metabolism of the abdominal viscera was much greater in the rats on 74 per cent casein. In them the apparent O_2 need of the viscera was

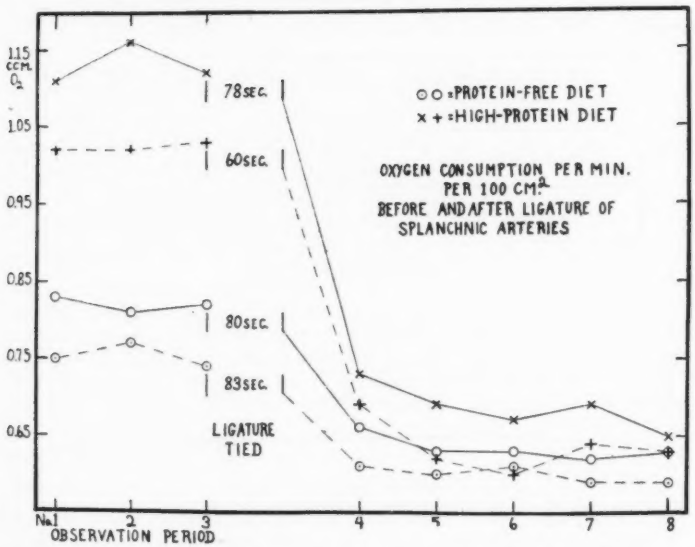

Fig. 3. Successive observation periods of individual rats (the two smallest rats in each group) before and after ligation of the blood supply of the abdominal viscera. Each observation period ends at the moment when the next period begins, and each represents the time required for the absorption of 2 cc. O_2 at atmospheric pressure and room temperature. The abrupt decrease in O_2 consumption even of the rats on high protein intake is evident. As the hepatic veins were not ligated some return of highly unsaturated blood may have occurred during the first few minutes due to respiratory movement of the abdominal viscera.

140 per cent greater than in those on 0 per cent casein, while the total metabolism was only 37 per cent greater and that of all the rest of the rats only 7 per cent greater in the high protein diet group. The decrease in oxygen consumption even in the rats on high protein diet was so abrupt, following splanchnic ligation, that it can scarcely be accounted for by disappearance of metabolites or stimulating substances liberated from the liver and acting on the other body cells (fig. 3). Part of the difference in metabolism of the abdominal viscera in the two groups may be ascribed to

differences in amount of hepatic tissue, since the 74 per cent casein group had 2.27 grams per 100 sq. cm. as compared with 1.97 grams of liver per 100 sq. cm. in the 0 per cent group. But even if the O_2 metabolism is converted into terms of O_2 per gram of liver, the 74 per cent group show an increase of 110 per cent over the visceral metabolism per gram of liver of the 0 per cent group.

The decrease in O_2 absorption after ligating the abdominal aorta and thus excluding all the tissues (except the testes) caudad to the renal arteries, was only 8 per cent greater in the 11 rats on a high protein diet than in the 10 rats on the low protein diet. The metabolism of the rats on 74 per cent casein, in this series, was 34 per cent greater than that of the rats on 0 per cent casein diet. It is evident, therefore, that the hind-quarters participate very little in the increased O_2 consumption due to protein metabolism. While the increase in oxygen consumption of the hind-quarters and, in the splanchnic ligation series, of the "eviscerated" rats on high protein diet is of about the same magnitude (7 and 8 per cent), the accuracy of the method is not such as to justify the conclusion that the metabolism of muscle and connective tissue of the rat is elevated during protein metabolism.

Discussion. These experiments on anesthetized rats indicate clearly that the various body tissues participate to very different degrees in the increased heat production and oxygen absorption which occurs following protein ingestion. While the elevation of total metabolism was 35 per cent on a high protein diet, the hind-quarter, the kidneys and the extra-abdominal tissues in general showed an increase of less than 10 per cent in oxygen requirement. The failure of renal ligation to modify the specific dynamic action of protein confirms Strieck's (8) observations on nephrectomized dogs. The liver and gastro-intestinal tract required more than twice as much oxygen following protein ingestion as they did after carbohydrate ingestion. As both groups of rats were eating the same bulk of food, it seems unlikely that the increase in metabolism in the abdominal viscera was due to increased metabolism of the gastro-intestinal tract. Wilhelmj and Mann (3), (4), (5) observed no increase in metabolism when glycine was administered to hepatectomized dogs. These experiments on rats suggest, therefore, that most, if not all, of the excess heat observed during protein metabolism is liberated in the liver. The livers of the rats on high protein diets were 15 per cent heavier, per unit of body surface, than those on protein free diet. Assuming that all of this increase is hypertrophy, the heat production of the liver, per gram of tissue, is still more than doubled by high protein diet. The conclusion that most of the heat resulting from the specific dynamic action of protein is liberated in the liver is not only in harmony with the observation of Mann and his co-workers that the liver is the site of deaminization and essential for the specific dynamic action of amino-acids, but also with the conclusions of Van Slyke

and Myer (9), (10), that amino-acids are accumulated in higher concentration and destroyed far more rapidly by hepatic tissue than by the muscles or other organs.

Of the previous studies of the effect of amino-acids on isolated tissue and on stimulation of hepatic metabolism by metabolites, only one is in accord with this conclusion. Freise (11) reported that the CO_2 production of perfused liver was increased as much as 50 per cent by lactic acid, glyceric acid and glucose, but not by galactose, glyoxylic, glycolic or acetic acids. In bits of excised liver Reinwein (12) observed no effect from phenylalanine, which has a marked specific dynamic action in the intact animal, and did observe an increased metabolism after adding glutamic acid, which has no specific dynamic action in intact animals. Rapport and Katz (13), perfusing isolated limbs of dogs, observed marked specific dynamic action when 1 per cent glycine was added, but this result was not controlled by perfusion with other substances in equivalent concentration and is in conflict with the observation of Wilhelmj and Mann, who found no increase in heat production in the absence of the liver.

Lusk (14, p. 276 et seq.) and Geelmuyden (15) have recently reviewed the theories concerning the specific dynamic action of amino-acids. Geelmuyden accepts the view that the excess heat represents the energy required for the transformation of amino-acids to glucose. Lusk draws no final conclusion, but points out (p. 304) that in the diabetic dog the excess heat production after giving alanine is very nearly that which would be required for its intermediary metabolism in being converted to glucose. Aubel (16) also reached the conclusion that the work needed for conversion to glucose accounts for the specific dynamic action of alanine. The fact that 85 per cent of the excess O_2 metabolism of rats on high protein diet is due to the increase in metabolism of the abdominal viscera can not easily be correlated with the theory that the amino-acids or some product of their intermediary metabolism stimulates cellular oxidation throughout the body. It does conform with the theory that the excess O_2 is used in converting these bodies to glucose, for it has been definitely shown that the liver is the only region where conversion of dihydroxyacetone to glucose (17) can occur and the chief site of glucose formation from the lactic acid in the blood (18), (19), (20). It therefore seems quite possible that the liver may be the chief or even the only region in which, not only the deaminization, but also the entire intermediary metabolism of amino-acids occurs.

SUMMARY

The rate of oxygen consumption of rats, fed on a diet containing 74 per cent casein and anesthetized with chloretone, was compared with that of controls fed on a diet containing no casein, immediately before and after ligating the blood supply of various regions of the body.

The O_2 consumption of the kidneys, in terms of O_2 per minute per 100 cm. of body surface, was practically the same in the rats on high and in those on a low protein intake, although the total O_2 intake of the former group was 35 per cent greater than the controls on low protein. Urinary secretion was not measured and no conclusions as to renal metabolism are to be drawn from these observations.

The O_2 consumption of the hind-quarters was only 8 per cent greater in the rats on the high protein diet, while that of the abdominal viscera was 141 per cent greater during protein than during carbohydrate feeding.

At least 85 per cent of the heat evolved as a result of the specific dynamic action of protein in chloretone-anesthetized rats is liberated in the abdominal viscera.

The significance of these facts is discussed in the light of previous observations by other workers, and it is concluded that the liver is probably the chief site, and possibly the only site, of the intermediary metabolism of those amino-acids which raise the metabolic rate, and that at least 80 per cent of the specific dynamic action of these acids is due to the increased energy liberated by the hepatic cells during protein digestion.

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THE EFFECT OF HISTAMINE UPON THE SECRETION OF GASTRIC PEPSIN

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In 1920 Popielski observed that the subcutaneous injection of histamine was followed by a copious gastric secretion. This observation paved the way for an extensive investigation of the action of this drug with the result that it is now being widely used as a test for gastric function. All investigators are in accord with the view that the gastric juice secreted in response to histamine stimulation is highly acid in character. The proteolytic activity of such juice has, however, received far less attention. In those studies in which pepsin determinations were made, the concentration of enzyme changed but slightly.

Rothlin and Gundlach (1921), in one of the earliest histamine studies, showed an increase of the proteolytic activity of the gastric juice obtained from a Pavlov pouch dog subsequent to histamine injection. Similar results have been observed in various clinical investigations (Carnot, Koskowski and Libert, 1922; Matheson and Ammon, 1923; Dobson, 1925; Rachon and Walawski, 1928). In none of these cases was the rise in peptic activity very marked. However, inasmuch as the volume of secretion was greatly augmented by the injection of histamine, the pepsin secretion in order to maintain its concentration, must have received a stimulus equal in intensity. In nearly all the investigations cited, Mett's tubes were employed to determine enzyme concentration.

Polland and Bloomfield, using an original and much more sensitive method for the determination of proteolytic activity (1929a), found that with increased volume of secretion there is a decrease in pepsin concentration (1929b). The increase in volume, however, is proportionally greater than the decrease in pepsin, with the result that the total amount of enzyme secreted per unit of time is increased by the action of histamine. Their experiments were performed on human subjects, the gastric juice being obtained by stomach tube. Quantitative technique under these circumstances is extremely difficult owing to the necessity of completely evacuating the stomach at definite intervals. In addition dilution of the stomach contents with saliva and loss through the pylorus are constant

sources of error. Babkin (1930), working with pouch dogs, made a careful study of the response of the gastric glands to histamine. The results of this investigation will be discussed later in this paper.

A simple and accurate method for the determination of peptic activity was recently developed in this laboratory (Gilman and Cowgill, 1930a). Utilizing this, the response of the gastric glands to histamine stimulation was studied quantitatively in order to determine the relative and absolute amounts of pepsin secreted into the gastric juice subsequent to histamine stimulation.

EXPERIMENTAL. Experiments were performed on dogs possessing gastric pouches so as to yield uncontaminated juice and to assure accurate collection. Both Pavlov and Heidenhain type pouches were employed. During the course of the experiment the animals remained standing, supported by a sling upon which they could rest their entire weight if desired. Before any experimental data were recorded, the animals were trained in the routine of the experiment, so that they remained quiet with no apparent signs of excitement or discomfort. Precautions were taken to guard the dogs against any possible external stimuli.

The gastric juice was collected by means of a soft rubber catheter inserted into the pouch opening and kept continually in place so that the secretion was drained from the pouch as rapidly as formed. All experiments were started at least 18 hours subsequent to feeding. Before the collection of samples the pouch was drained for 5 minutes to assure complete evacuation of all its contents. Following this the basal fasting secretion of the pouch was collected until sufficient was obtained for analysis. At this point the animal received a subsutaneous injection of histamine (0.2 mgm. per kilo) following which samples were collected in successive 15-minute periods until the secretion rate returned to a level approximating that of the fasting stomach. In addition to the examination for pepsin the samples were analyzed for free and combined HCl by titration with N/50 NaOH using Töpfer's reagent and phenolphthalein respectively as indicators. Total chlorides were also determined by the method of Van Slyke (1923).

RESULTS. *Volume:* As has been reported from all previous histamine studies, the volume of juice secreted rapidly increases, reaching its maximum from 30 to 45 minutes after injection and then slowly diminishing.

Acid and total chloride: With increased rate of secretion there is an increase in free and total acidity, reaching a maximum value coincident with the maximum rate of secretion and tending to maintain this value throughout the remainder of the experiment. Total chlorides tend to remain constant during the entire course of the secretion. The maximum value for total HCl is usually from 145 to 150 MEq. Chlorides remain fairly constant at 160 to 165 MEq. A more detailed account of the acid and chloride secretion of the stomach is reserved for a later paper.

Pepsin: With increased volume of secretion the concentration of pepsin shows a definite marked decrease. The fasting basal secretion of the stomach invariably has a concentration of pepsin greater than that of any sample secreted in response to a histamine stimulation. The greater the volume of the sample, the lower is the concentration of pepsin. When the gastric juice is flowing at its maximum rate proteolytic potency is at a minimum. After the peak of the stimulation is passed, and the rate of volume flow diminishes, the concentration of pepsin slowly increases, approaching the level of the basal fasting secretion. This observation is illustrated graphically in figure 1. Identical results were obtained in the seven dogs subjected to the experiment, four having Pavlov and three having Heidenhain type pouches.

The decrease in concentration of pepsin with increased volume of secretion would seem to indicate that the pepsin secreting glands had received no stimulus from the histamine injection. In order to approach this question more quantitatively, the total amount of pepsin secreted per unit of time was calculated by multiplying the pepsin concentration by the rate of flow. If the secretion of the enzyme had received no stimulation we should expect this value to remain constant throughout the course of the experiment. The results are graphically illustrated in figure 2. Here again as in figure 1 an increased volume of flow is associated with a decreased concentration of pepsin. However, immediately after the injection of histamine, the increase in volume is proportionally greater than the decrease in pepsin concentration with the result that the total amount of pepsin secreted per unit of time is substantially increased. This augmented pepsin secretion is only noticed in the first sample collected subsequent to stimulation. Following this sudden rise, the total pepsin rapidly falls, and by the time the volume of secretion has reached its maximum the total amount of enzyme secreted per unit of time has reached a level as low or lower than that of the sample collected before the histamine was injected.

At first glance it would seem that the pepsin-secreting glands had received a definite and positive stimulus. However, it must be observed that at the time when the stimulus from the histamine is obviously at its maximum, namely, when the rate of volume secretion and the concentration of HCl is the greatest, the total amount of enzyme secreted per unit of time is no larger than before the stimulus was given. If the histamine is acting as an enzyme secretagogue it stimulates the pepsin secreting glands before the effect upon the acid secreting glands has reached its maximum. The rapidity of the response of the enzyme secretion and the marked delay before the volume of flow reaches its peak might be explained by a nervous and humoral transmission of the respective stimuli. This is negated however by the fact that both Pavlov and Heidenhain dogs respond in exactly

the same manner. Some other explanation must be sought, therefore, to explain the time discrepancy between the maxima of these two secretion curves, and to account for the transitory nature of the increased pepsin secretion, bearing in mind that it is only the total output of enzyme that is augmented, the concentration of pepsin showing a decrease.

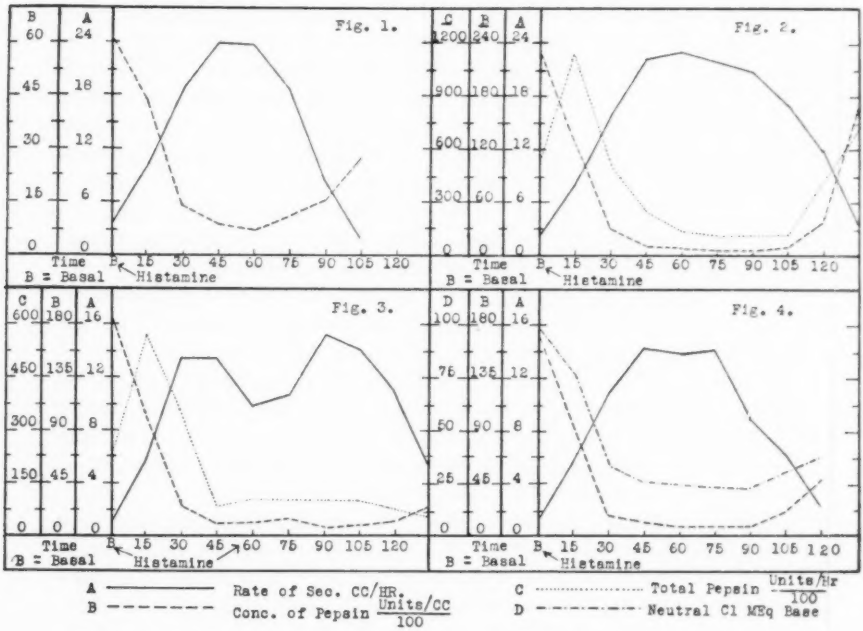
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
B = Basal
Histamine
Time
A ——— Rate of Sec. CC/HR.
B - - - - - - - Conc. of Pepsin Units/CC / 100
C ..... Total Pepsin Units/Hr / 100
D - · - · - · - Neutral Cl MEq Base

Figs. 1–4. The inverse relation of pepsin concentration to the volume rate of secretion is illustrated in figure 1. In figure 2 is shown the great increase in the total amount of pepsin contained in the first sample of gastric juice obtained after injection of histamine. The effects of successive injections of histamine are illustrated in figure 3. The positive influence of each injection on the volume rate of flow is clearly evident. Note the sudden increase in the total pepsin present in the first sample subsequent to the initial histamine injection, as contrasted with the constancy of pepsin content in the samples collected after the second injection. In figure 4 is shown the parallelism between the concentrations of pepsin and neutral chloride.

One of the characteristics of the fasting gastric secretion is the presence of large quantities of mucus. This makes the quantitative drainage of this sample difficult, and that some of this mucoid material remains adhering to the walls of the stomach is inevitable. In addition the furrows of the mucosa may retain an appreciable quantity of juice, and the lumens

of the glands must undoubtedly be filled with the characteristic pepsin-rich fluid. Following histamine stimulation there is a sudden secretion of gastric juice, more acid in nature but much more dilute in enzyme. As a result the highly proteolytic residue of the fasting secretion is washed from the lumen of the glands and the furrows of the mucosa and included in the first sample subsequent to histamine injection. Thus by a purely mechanical lavage the total pepsin content of this sample is increased, although there is a decrease in pepsin concentration.

To establish this conclusion experiments were performed in which double stimulations were employed. After the response to the first injection of histamine had reached its peak, a second injection, equal to the first, was made. If the increase in total pepsin observed in figure 2 was actually due to a stimulation, a second injection of histamine, given when the total pepsin had reached its low level, should again cause a sudden sharp rise. If on the other hand the increased pepsin was due purely to a lavage, a second injection of histamine would have no effect inasmuch as the stomach has already been washed free of its residual, enzyme-rich, fasting secretion. Ten such experiments were performed on four dogs. Typical results are illustrated in figure 3.

This graph exhibits as before, the sudden rise in total pepsin in the first sample obtained subsequent to histamine injection. This value rapidly decreases, falling to a level lower than that of the fasting juice 45 minutes subsequent to injection. Sixty minutes after the first administration of histamine, at a time when the effect of this stimulation was beginning to diminish as evidenced by the decreased volume of flow, a second injection of histamine was made. That this subsequent stimulus had a definite effect is evidenced by another increase in secretion rate, the maximum being greater than that induced by the first administration of the drug. The rate of enzyme secretion however is uninfluenced, continuing at the same level. One may conclude, therefore, that the increase in the total pepsin content of the first sample obtained after histamine is a result of a mechanical washing of the residual enzyme rather than a true stimulation of the pepsin secretory processes. The enzyme secreting cells continue to function, but at a constant basal rate, indicative of the fact that they have not received stimulation.

It is of interest in an experiment of this kind to attempt to correlate the secretion of enzyme with the other constituents of the gastric juice. Thus Polland and Bloomfield (1929b) and Babkin (1930) observed that the nitrogen content of their gastric samples runs practically parallel with the digestive power. MacLean and Griffiths (1928) have reported a parallelism between pepsin and neutral chloride concentration. In our study the nitrogen content of the samples was not determined. However, in all experiments the value for neutral chlorides was calculated from the

difference between total and acid chloride. Furthermore, in several series the value of neutral chloride was obtained by determination of fixed base, the method of Stadie and Ross (1925) being employed. As shown in figure 4, in which values for neutral Cl were obtained by direct determination of fixed base, the observations of MacLean and Griffiths are supported.

Discussion. Subsequent to the appearance of the preliminary report of this investigation (Gilman and Cowgill, 1930b) our attention was called by a personal communication from Doctor Babkin to experiments being conducted in his laboratory, a résumé of which was published prior to our first report. It is gratifying to note the close agreement between these separate investigations. Babkin and associates (1930), using Mett's tubes, observed the same decrease in pepsin concentration following histamine stimulation and conclude that "Histamine, therefore, has no 'trophic' effect on peptic and mucoid cells, or only a very insignificant one. It activates chiefly the parietal cells, which are supposed to produce the hydrochloric acid in the juice, or its precursors." This statement is in perfect accord with the experimental evidence herein presented.

It is of interest, in view of the above statement, to return to a discussion of the obvious parallelism between neutral chlorides and pepsin concentration. One cannot conclude from this observation that neutral chloride and pepsin are secreted in conjunction with one another. However, it seems obvious that the production of neither is stimulated by histamine, both secretions continuing at a basal level, which accounts for the parallel course of their concentration. Thus the secretion of fixed base and that of HCl are the products of two distinct sets of glands, and the eventual pH of the pure secretion as obtained from a pouch would depend upon the relative secretion rates of these two components. In the fasting secretion of the stomach the secretion of base almost equals that of the acid, with the result that the reaction of the gastric contents approaches neutrality. Subsequent to histamine stimulation, however, the secretion of the basic elements of the gastric juice is so insignificant in comparison with that of the acid that practically all the Cl of the juice is present as HCl. This would support the theory of Pavlov and the recent work of Hollander and Cowgill (1931), namely, that the hydrochloric acid of the gastric juice is secreted at a constant acidity, any variations in acidity being due to fluctuation of the independent basic constituents.

The fact that the enzyme secretion of the gastric juice is not stimulated by histamine does not reduce the value of this drug for use in tests of gastric function. However, as Babkin points out, one is only determining the capabilities of the acid-secreting glands to respond to a stimulus. The pepsin secretion proceeds at its basal level, and one can only determine the potential enzyme-secreting power of the stomach by superimposing another suitable stimulus. The application of such a stimulus to gastric function tests is now being carried out in Babkin's laboratory.

The independence of the secretory rate of the various constituents of the gastric juice should also again be emphasized. Very often in the literature one notes the inference that the proteolytic activity of gastric samples must be high inasmuch as the acid content of the juice was high. That this is not necessarily true is now obvious. In this study and in Babkin's investigation, the highest proteolytic power was associated with low acidities. Conversely high acidities may be associated with very low pepsin concentration. We are dealing with two separate processes, independent in their activities and influenced by individual stimuli.

CONCLUSIONS

Studies of the response of the pepsin secreting glands to histamine stimulation were made in seven gastric pouch dogs of the Pavlov and Heidenhain type. With increased volume of secretion in response to histamine stimulation there was a relative decrease in pepsin concentration indicative of the fact that the histamine had not stimulated pepsin secretion. A parallelism between pepsin and neutral chloride concentration in the gastric juice was also observed.

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FUNCTIONAL AND HISTOLOGIC STUDIES OF THE EFFECT OF FAT INGESTION UPON THE NORMAL AND DAMAGED LIVER

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The important relation of the liver to fat metabolism in the normal animal, and the fact that the liver reacts to most forms of acute injury by an excessive accumulation of fat in its cells led us to investigate the influence of fat administration upon bile pigment metabolism and upon the bromsulphalein liver function test.

The experiments of Leathes and Meyer-Wedell (1), Coope and Mottram (2), Roper (3), and Bodo and Scheffer (4), demonstrate that large accumulations of fat in the liver can occur under conditions approaching physiological, but there have been no observations to show that this fat deposition interferes with any other functions of the liver cells. We believe that just as the ingestion of particulate matter by the reticuloendothelial cells can temporarily derange the physiology of this system, so can an excess of fat in a cell interfere with its activity.

Method. In earlier experiments lipemia was produced by feedings of mutton fat, but it was found more satisfactory to administer cream either by mouth or stomach tube. The methods employed for studying functional activity of the liver were the bromsulphalein test of Rosenthal and White (5), the estimation of bilirubin in the blood (Cutten, Emerson and Woodruff, 6), and of urobilinogen in the urine (Wallace and Diamond, 7). Lipemia interferes with the colorimetric comparison of dye and bile pigment in the serum. We have not found acetone satisfactory for extraction of bromsulphalein from serum as did Bloom (8) with phenoltetrachlorphthalein. The method finally adopted was to place the lipemic serum in narrow test tubes and stand them upright overnight in the cold room, when the fat was found to accumulate at the top of the tube. If separation was incomplete centrifugation at high speed while cold in most instances rendered the serum clear enough for estimations of the dye. The sera were not often made clear enough for colorimetric comparisons of bilirubin and in these experiments there was not always a

sufficient bilirubinemia to employ the van den Bergh reaction for its estimation. It was often necessary to employ approximate comparisons

TABLE 1

The effect of lipemia upon the removal of bromsulphalein from the blood

Lipemia was produced by feeding mutton fat or was found to exist spontaneously.

DOG	LIPEMIA	BROMSULPHALEIN IN SERUM		
		5 minutes	10 minutes	15 minutes
		per cent	*per cent*	*per cent*
XC	0	20	3	
	+++	28	9	
XM	0	35	14	5
	+	40	18	11
XO	0	10	2	
	+	18	5	
1	0			Trace
	+			5
3	0			Trace
	+			5
4	0			Trace
	++			8

TABLE 2

The effect of fat ingestion on the bromsulphalein test. Dog fed mutton fat

	FAT IN DIET (DAY PREVIOUS)	LIPEMIA	BROMSULPHALEIN IN SERUM		
			3 minutes	5 minutes	10 minutes
	gm.		*per cent*	*per cent*	*per cent*
May 27	0	0	43	18	2.8
June 3	0	0	40	21	
June 16	0	0	44	22	3
June 17	0	0	45	17	
June 22	300	+	53		5
June 23	600	+++	70	28	9
June 24	600	+++	60	25	5
June 28	0	0	50	22	7
June 29	0	0	43	20	

with potassium dichromate as a standard, using test tubes in a comparator box. A fair degree of accuracy was obtained in this way.

Results. In a group of normal dogs lipemia was associated with slight

degrees of dye retention (table 1). Dog XC was fed mutton fat for a period of five days in addition to 100 to 200 grams of lean meat daily. Definite dye retention was produced during the lipemic phase (table 2). The dye test usually returned to normal parallel with the disappearance of the lipemia.

The impairment of function as reflected by the dye test can reach con-

TABLE 3

The effect of oral administration of cream to dogs with damaged livers

Considerable impairment of functional activity as shown by the bromsulphalein test. In one dog the lipemia was spontaneous (no fat in diet).

DOG WEIGHT	HISTORY OF LIVER INJURY	AMOUNT OF CREAM FED	LIPEMIA	RETENTION OF BROMSULPHALEIN IN BLOOD PRIOR TO LIPEMIA	RETENTION OF BROMSULPHALEIN IN BLOOD DURING LIPEMIA
kgm.		*cc.*		*per cent*	*per cent*
11.5	2 hrs. $CHCl_3$ 19 days previously	400	+++	10	20
9.5	2 hrs. $CHCl_3$ 19 days previously	375	+++	6	19
		200	+		16
12	2 hrs. $CHCl_3$ 20 days previously	None*	+++	10	30
7.3	2 hrs. $CHCl_3$ 19 days previously	300	+++	10	40
		300	+++		30
9.3	1½ hrs. $CHCl_3$ 18 days previously	350	++++	10	40
		200	+++		37
		0†	+		27
6	3 cc./kilo CCl_4 12 days previously	350	++++	8	28
		0†	0		23
8	4 cc./kilo CCl_4 12 days previously	350	+++	9	16

* Spontaneous lipemia.

† Twenty-four hours after cream feeding.

siderable magnitude when cream is fed to an animal with a damaged liver. Five animals were employed that had been anesthetized with chloroform for two hours, 18 to 20 days previously. At the time lipemia was produced, pigment metabolism had returned to normal and morphologically such a liver would show practically complete repair, although functionally some degree of dye retention persists until the fifth or sixth week (9).

Carbon tetrachloride was used to produce liver injury in two additional

dogs (table 3). Under these conditions the feeding of fat produced a marked increase in the retention of dye. The amount of bromsulphalein in the serum 15 minutes after injection ranged from 7 to 15 per cent before the lipemic state. During the height of lipemia there was 20 to 40 per cent retention (charts 1, 2).

Some of the dogs with livers damaged by chloroform had also been employed to study the effects of alcohol under these conditions, and it is seen in chart 2 that 40 cc. of 20 per cent cream per kilo (by mouth) had a much more marked effect than 2 cc. per kilo of 95 per cent alcohol.

The dye retention returned to its previous level when the lipemia

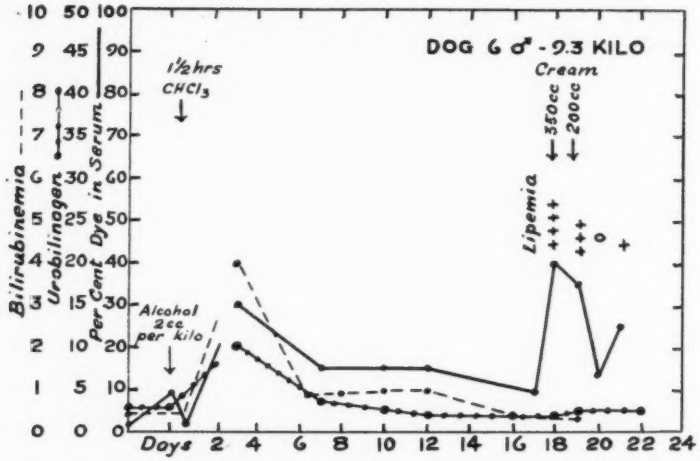

Chart 1. Experimental lipemia in a dog with incomplete recovery from chloroform poisoning. No effect on bile pigment metabolism but marked effect on dye retention. The percentage of dye in the serum represents that which remains 15 minutes after the intravenous injection of bromsulphalein.

disappeared, except in one dog with whom some impairment was present for 24 hours following the lipemia.

In no instance have we been able to demonstrate any disturbance in bile pigment metabolism in these dogs. Very slight changes in the bilirubin concentration in the serum may have been obscured by the lipemia, but the urobilinogen excretion in the urine was not affected.

What is the significance of the retention of bromsulphalein in the blood caused by the ingestion of fat? It is not a question of solubility of the dye in the lipoids of the blood, for it is insoluble in fat solvents such as benzene, chloroform, and xylol, and further, the fats are largely removed from the serum before estimations of dye are made. We believe it sig-

nifies disturbance within the liver and this belief is supported by the much more marked retention present when the liver is damaged. We have previously found that other substances which cause transitory liver damage, such as alcohol and ether (9), will bring about a retention of dye without any alteration in pigment metabolism.

We do not agree with Shellong and Eisler that the dye is removed from the blood by the entire reticulo-endothelial system. These authors

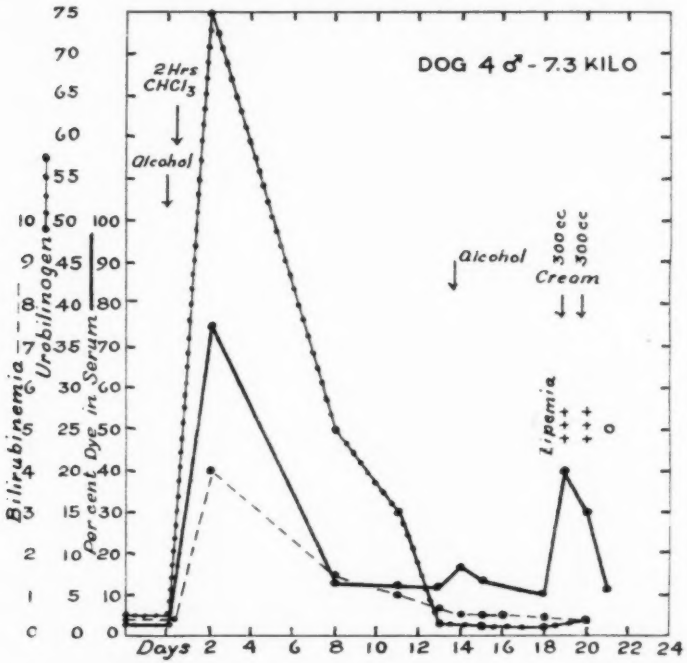

Chart 2. Fat administration to dog with liver previously damaged with chloroform. Considerable disturbance of liver function shown by the bromsulphalein test. No disturbance of bile pigment metabolism. Two cubic centimeters per kilo of 95 per cent alcohol had a less marked effect under these circumstances.

(10) found dye retention in splenectomized rabbits and rabbits injected with India ink. They used phenoltetrachlorphthalein and Asorubin S. and employed only the qualitative "ring test" for their estimations. We have repeated their experiments and found that splenectomy or blockage of the reticulo-endothelial apparatus with quartz does not alter the removal of bromsulphalein from the blood (table 4). Their results may be explained by the fact that phenoltetrachlorphthalein as-

it circulates in the blood (in the colorless form) is insoluble and therefore behaves as finely particulate matter, while bromsulphalein is soluble and dialyzable in its acid state. We have previously demonstrated (5) that bromsulphalein is specifically taken up by the liver, while phenoltetrachlorphthalein is not. It must be pointed out that the relation of certain of the reticulo-endothelial cells, the Kupffer cells, to absorption from the blood and excretion by the liver of bile pigments and dye stuffs has not been established. Regardless of the rôle played by the Kupffer cells, our experiments lead us to believe that the dye retention expresses a temporary derangement of the liver caused by the burden which fat administration places on it. Lamson (11) has found that cream increases the toxicity of carbon tetrachloride orally administered, and it would be worth while to repeat such studies when this drug is given parenterally and the cream by mouth. Our results would indicate that the effect of

TABLE 4

The negligible effect of splenectomy or blockade of the reticulo-endothelial apparatus by colloidal quartz upon the removal of bromsulphalein from the blood

	BROMSULPHALEIN IN SERUM		
	5 minutes	15 minutes	30 minutes
	per cent	*per cent*	
Rabbits, normal	15–30	Trace to 3	0 to Trace
½ hour after splenectomy	32	2	S.P.T.
½ hour after splenectomy	35	Trace	S.P.T.
3 hours after 15 cc. quartz intravenously	15	Trace	
2 hours after 10 cc. quartz intravenously	33	7	
4 hours after	20	Trace	

cream on the liver, as well as any influence of the cream in increasing the absorption of carbon tetrachloride, must be considered in the mechanism of this increase in toxicity.

Neither is it thoroughly established what part is played by the reticulo-endothelial system in the removal of fats from the blood following a physiological lipemia. Ziegler (12) from his experiments on guinea pigs, concludes that the endothelial cells of the liver and spleen regularly take up fat under these conditions, but more recent workers overlook or do not accept Ziegler's experiments. Oppenheimer and Fishberg (13) state that in pathologic lipemia the reticulo-endothelial cells often store all types of circulating lipoids. Anitschkow (14) fed cholesterol dissolved in sunflower oil to rabbits, over a long period of time, and found that it was stored in the reticulo-endothelial system.

We have produced lipemia by feeding dogs 25 cc. to 75 cc. per kilo of cream (20 per cent fat) daily in addition to approximately 100 grams of

lean meat. The cream was given in place of water so that it was drunk throughout the day. This insured a maximum degree of digestion and absorption. Varying degrees of sustained lipemia were produced in all cases. Eight dogs were fed cream in this manner for periods of 2 to 20 days. Five dogs were used as controls and were kept on a diet of lean meat and bread for 2 weeks to 2 months. Three of the control dogs were killed 12 hours after the last feeding; one was starved 2 days and another was starved 4 days prior to sacrifice.

Histologic studies were made for the presence and differentiation of fats in various organs by the use of fat stains, sudan, scarlet red, osmic acid, and nile blue, and by the use of Nicol prisms to determine their refractive characteristics.

There were qualitative increases above the controls in the fat content of the liver epithelium, produced by fat feeding, but no appreciable uptake of fat was found in the Kupffer cells.

The relation of the bile duct epithelium to fat metabolism was brought out to us in a surprising manner. Considerable quantities of fat droplets were present in the bile duct and gall bladder epithelium in all of the cream fed dogs. Fat was often present to a sufficient extent to cause the bile ducts to stand out å bright red when stained with Sudan IV. At first this was believed to be a deposition of the recently ingested fat but an investigation of the control dogs showed it to occur to practically the same degree in four of the five dogs examined. In both series there were variations in the degree of fat deposition which we have been unable to correlate with the intake of food or the feeding of fat. In one of the control dogs there was very little fat in the epithelium of the biliary tract. Most of the fat in the bile duct epithelium occurs as neutral fat or fatty acids, although some doubly refractive material is present. Its distribution is widespread, for it is present in the small and large bile ducts and also in the gall bladder epithelium. The distribution of the fat in these cells is chiefly confined to their basal two-thirds. The following protocols which are typical of the findings in 12 of the 13 dogs studied histologically, will illustrate the presence of fat in the biliary epithelium, and its absence from the Kupffer cells.

Dog 24. Male, 6 kilos. Four hundred cubic centimeters cream (20 per cent fat) by stomach tube, in addition to 100 grams lean meat, daily, for 2 days. Autopsied 7 hours after second feeding. Blood serum lipemic +++.

Liver: The liver cells are generally large, finely granular and moth-eaten in appearance, with irregular, partly clear areas. Their nuclei are well stained, of normal size and with normal appearing nucleoli. The capillaries are narrowed and anemic. The endothelial cells are thin and flat.

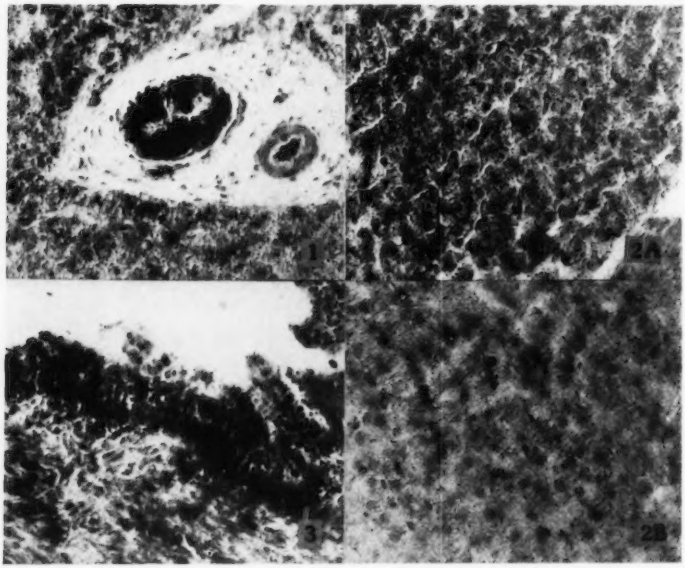

Photo 1. Fat in the epithelium of a small bile duct and in the liver cells. X 200. Hemotoxylin and Sudan IV, B filter. Dog 27.

Photo 2. (A) Fat in the liver cells of dog 24 fed 400 cc. of cream daily for two days. (B) The absence of fat in the liver cells in dog 91, on a fat free diet for two months. X 200. Hemotoxylin and Sudan IV, B filter.

Photo 3. Fat in a large bile duct of dog 24.

The bile duct epithelium contains small globules of fat invisible with crossed Nicol prisms and staining pink with nile blue sulphate, a few other globules staining blue with nile blue, and others bright with crossed Nicol prisms.

Here and there in the liver cells are masses of doubly refractive material stained deep blue by nile blue, as well as more numerous minute doubly refractive droplets which are unstained, or pale pink with nile blue.

Dog 27. Male police dog, 16 kilos. Seven hundred fifty cubic centimeters cream fed daily for 3 days. Autopsied at end of third day. Blood intensely lipemic. The liver has a greyish fatty appearance. Microscopically, the cytoplasm of the liver cells present a moth-eaten appearance, irregular granules of stained material remaining among likewise irregular clear spaces. Their nuclei are everywhere well stained, their nucleoli of normal size and intensity of staining. The bile duct epithelium often shows coarse sharply contoured vacuoles around the nucleus. The

free margins of the cells show a zone of nonvacuolated cytoplasm one-third the height of the cell.

In frozen sections the opaque moth-eaten appearance of the cells is retained, the clear spaces largely remaining empty when sudan IV is used. There are in the liver cells moderate numbers of small oil globules and irregular sudanophil particles. Rare fat globules are seen in the reticulo-endothelial cells. The capillary spaces are narrow. The coarse vacuoles seen about the nuclei in the bile ducts are replaced by intensely red stained fat (Sudan IV).

Dog 18. Male terrier, 10 kilos. Three hundred cubic centimeters cream in addition to 100 grams of lean meat, daily for 21 days.

Liver: In paraffin sections the liver cells are essentially similar in appearance to those of dog 27. The endothelial cells are thin and flattened. The bile duct epithelium is vacuolated, and contains rather small fat droplets. The liver cells contain fine fat droplets. The portal veins show numerous fine fat droplets in their lumina.

Dog 91. Female terrier, 10 kilos. Lean meat and bread diet for two months; no fat.

Liver: The cytoplasm of the liver cells shows the moth-eaten reticulo-granular appearance seen in well nourished dogs, but no fat. The bile duct epithelium shows coarse epinuclear vacuoles and in sudan IV preparations these epithelial cells are filled with fine and medium fat globules, only the cuticular margin and the nuclei being free. This fat stains red with sudan IV, clear pink, violet, or deep blue with nile blue sulphate, is blackened by osmic acid after bichromate, and includes numerous angular epinuclear doubly refractive crystals. The doubly refractive lipoids are soluble in cold acetone.

Gall bladder: Fine to medium vacuolation of the epithelium chiefly in the epinuclear zone. Fat droplets are most numerous in the epinuclear zone and are also seen basally and up almost to the upper fourth of the cells. This fat stains red with sudan IV, black with osmic acid, faint pink, violet, or blue with nile blue sulphate, and includes numerous droplets and elongate needles of doubly refractive lipoid.

Dog 104. Diet of lean meat and bread for three weeks; no fat. Starved for 96 hours prior to autopsy.

Liver: Liver cells present granular cytoplasm. Rather marked filling of liver cells with fine fat droplets. The last is quite irregular in distribution and often the cells adjoining the portal connective tissue show less fat than those farther out. The larger bile ducts show marked infiltration of their epithelial cells with fat.

Gall bladder: There are fine fat droplets beneath and above the nucleus in the epithelium.

Does the presence of fat in the biliary epithelium represent a resorption

of fat from the bile? Virchow (15) in 1857 found fat in the gall bladders of humans, dogs, and cats, and postulated such a view. Policard (16) described fat in the gall bladder epithelium of dogs and cats and expressed the belief that fat absorption occurred in the same manner in the gall bladder as in the intestinal mucosa. Numerous investigators have injected fats, dye stuffs and crystalloids into the gall bladder and demonstrated a subsequent absorption. An excellent review is given by Winkenwerder (17), who demonstrated that potassium ferrocyanide with ferric ammonium citrate could be absorbed through the epithelium of the gall bladder and larger bile ducts. Our studies have revealed that *in the dog* normally and after fat feeding a considerable amount of fat is distributed throughout the epithelium of the entire biliary tract. As a better knowledge of the mechanism of secretion of bile is acquired, it is possible that a process of secretion and intrahepatic resorption of some of its constituents will be found to occur, analogous to certain phases of urine secretion.

SUMMARY

Lipemia, either spontaneous or experimentally produced, causes a slight impairment of liver activity as shown by a retention of bromsulphalein in the blood. This amounts to 5 to 11 per cent 15 minutes after injection of the dye. There is no increase in urobilinogen excretion in the urine and no elevation of bilirubin in the blood.

If lipemia is produced in a dog whose liver has been previously damaged with chloroform or carbon tetrachloride, the retention of bromsulphalein amounts to 20 to 40 per cent.

Splenectomy and reticulo-endothelial cell blockade produce negligible disturbances in the removal of bromsulphalein from the blood. This is further evidence to that already obtained to show that this dye is removed from the blood almost entirely by the liver.

Varying degrees of sustained lipemia were produced by feeding large amounts of cream to 8 dogs for 1 to 20 days. No appreciable amount of fat was taken up by the Kupffer cells as a result of these feedings.

There are normally present in dogs considerable accumulations of fat in the biliary epithelium, both in the intrahepatic ducts and in the gall bladder. No correlation could be made between the quantity of fat in the bile duct epithelium and diet, in dogs on a high fat diet, dogs on a low fat diet, or dogs that had been starved for 1 to 4 days.

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STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM

XB. THE EFFECT OF INTRAVENOUS CALCIUM CHLORIDE ON PERISTALSIS FOLLOWING INTESTINAL OBSTRUCTION IN DOGS

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It has been observed clinically that the intravenous injection of 15 cc. of a 5 per cent solution of calcium chloride tends to inhibit the pain of biliary and renal colic in humans (1). The relief from pain may be transient or may last for several hours. The results are constant and dramatic in the relief of the pain of lead colic. In order to study the mechanism by which calcium chloride inhibits pain arising in organs composed of smooth muscle, its effect was studied upon intestinal peristalsis in dogs.

METHOD. Changes in pressure in the bowel, following intestinal obstruction, were recorded by means of a balloon connected to a water manometer. The intestinal obstruction was produced in dogs by a two-stage operation. All operations were performed aseptically under ether anesthesia. At the first operation a midline incision about 7 cm. long was made low in the abdomen. The peritoneum was opened and the lower 15 cm. of the small intestine was isolated and brought through the wound. First the peritoneum and then the abdominal musculature was sewed together through the opening in the mesentery, thus placing about 5 cm. of the small intestine above the abdominal muscular layer so the peritoneum would not be infected by leakage of intestinal contents subsequent to the second operation. The skin was then sewed together over the intestine. After two days, adhesions bound the peritoneum to the small intestine preventing peritoneal infection during and after the second operation. The skin was reopened after two days and a 1 cm. incision was made lengthwise in the wall of the intestine. A rubber balloon firmly attached to a mushroom catheter was inserted into the proximal end of the intestine by means of a catheter director. The edges of the intestinal wound were sewed to the catheter just above the attachment of the ballon and the skin closed. After 24 to 48 hours peristalsis may be recorded by filling the balloon with water and attaching the catheter to a water manometer. The

peristalsis forces the mushroom catheter down the intestine to the point where it emerges between the abdominal muscles but these prevent the balloon and catheter from being extruded.

RESULTS. To observe the effect of intravenous calcium chloride alone on intestinal peristalsis after obstruction, the balloon was first inserted into a dog weighing 11.8 kilos by the method described above. Five days later records of peristaltic movements were made. Regular contractions occurred at intervals of 3 to 5 minutes raising the pressure as high as 2.4 cm. of water. Since the usual dose of calcium chloride administered to humans intravenously is 0.010 gram per kilo, the same initial dose was given to the dogs. As it was without effect the dose was increased and finally doubled, i.e., 0.020 gram per kilo. Although the larger dose caused vomiting, there was no prolonged effect on the height or frequency of the peristaltic movements. The same results were obtained on another animal in a subsequent experiment.

Several observations have been recorded on the action of calcium in isolated smooth muscle. Thus Vanysek (2) reports that calcium salts exert a relaxation of the bowel. When calcium chloride was added to the solution bathing the isolated intestinal muscles of cats, it caused a relaxation of the longitudinal muscle but a stimulation of the circular muscle. Kennedy (3) found that doubling the calcium content of Dale's solution inhibits the rhythmic contractions of the isolated rat's uterus. It is not surprising that our results on the intestine of living animals is at variance with these results, for our effect upon the blood calcium concentration must have been relatively slight. It is also to be noted that we have used a different species of animal, and the variation of pharmacological effects in different species is well known.

McIntosh and Owens (4) made observations on the increased peristalsis in dogs with intestinal obstruction following the administration of eserine. As calcium chloride in the given doses had no inhibitory effect on the normal peristaltic movements after obstruction alone, its effects were next studied on the increased peristalsis produced by eserine. Eserine sulphate (1.3 mgm.) caused a marked increase in intestinal peristalsis, the pressure rising to 5.4 cm. of water. The effect of surgical pituitrin in doses of 0.1 to 0.4 cc. was much less marked than eserine, hence it was used in only one experiment. Calcium chloride in doses as large as 0.022 gram per kilo had no effect on the peristalsis produced by eserine. Similar results were obtained on three animals. When peristalsis was stimulated by eserine, the intravenous injection of 2 mgm. of atropine completely stopped perisistaltic movement within two minutes.

It has been repeatedly observed by one of us that 15 cc. of a 5 per cent solution of calcium chloride injected intravenously in man stops the pain of lead colic almost at once. An effort was therefore made to produce lead

colic in dogs by the intravenous administration of colloidal lead. Thirty to fifty milligrams were injected at intervals of 1 to 3 days until 525 mgm. had been given intravenously. An additional 240 mgm. were given intratracheally to the animal used to make the record shown in the figure. Since this animal gave no indication of pain it is probable that there was no actual lead colic. Calcium chloride in doses as large as 0.078 gram per kilo was then injected intravenously. Peristaltic movement continued though the high pressure waves disappeared for about thirty minutes. The animal was profoundly affected as shown by vomiting and abdominal respiratory movements (see fig. 1). Smaller doses were without effect.

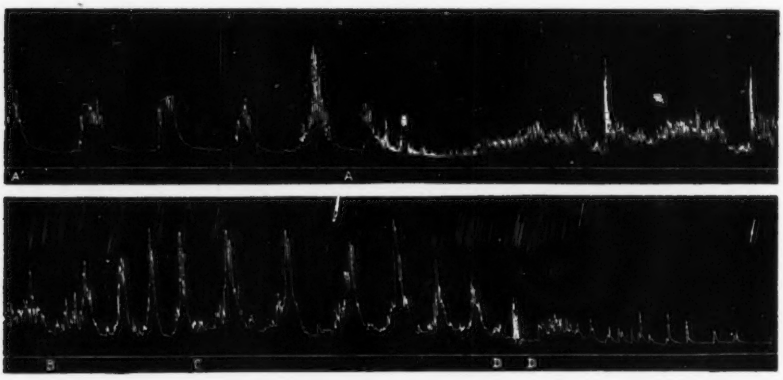

Fig. 1. Intestinal peristalsis in the dog. The effect of calcium chloride after the prolonged administration of 0.765 gram of colloidal lead and after intestinal obstruction. *A'*, 15 minutes before injection. *A*, 10 cc. 5 per cent $CaCl_2$ intravenously. *B*, 30 minutes after injection. *C*, 45 minutes after injection. *D*, 10 cc. 5 per cent $CaCl_2$ injected again, one hour after first injection.

It may be that calcium chloride stops the pain of lead colic in man by inhibiting peristalsis as suggested by Vanysek's results. Since the dogs did not develop lead colic we cannot say that it does not relax the intestinal spasm that accompanies that condition. At least the presence of large amounts of lead had no marked effect on the type of peristalsis produced or on the lack of results which followed the use of calcium chloride.

CONCLUSIONS

No demonstrable change either in peristalsis or intestinal muscle tone could be found in dogs after the intravenous injection of small or large doses of calcium chloride.

The observations included the effect on:

1. Simple intestinal obstruction.
2. The stimulating effect of eserine.
3. The production of chronic lead intoxication.

The experiments described in this paper leave the problem of explaining the favorable action of calcium chloride in smooth muscle colic unanswered.

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THE EFFECTS OF DEFICIENT WATER-INTAKE ON THE GROWTH OF THE RAT

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Although various investigators have shown that a deficient water-intake prevents growth, the nature of this effect remains somewhat uncertain. Some have noted that under this condition the food-intake is also decreased, and that this may account for the inhibited growth. The present experiments were devised to throw light on this question by subjecting young rats with equal food consumption to a varied intake of water over extensive periods of time. Some observations were made also on the weight and water content of the various organs.

MATERIAL AND METHODS. Rats from the colony in the Institute of Anatomy were used in the experiments. Series A included a single litter of 5 female albino rats, Mus (Rattus) norwegicus albinus. Series B included 7 hybrid (pied) rats, 4 males in one litter and 3 females in another. The initial and final ages and body weights are shown in table 1. In designating the individual rats, "A" or "B" indicates the series, "c" or "t" whether control or test, "m" or "f" the sex. The final number indicates the individual.

The 12 rats form 3 groups. 1. Two rats were used as normal controls, with unrestricted food and water. 2. Seven, designated as "test rats," were given food ad libitum, but merely sufficient water to hold the body weight as nearly constant as possible. 3. Three rats, designated as "restricted food" controls, were each fed the same amount daily as was voluntarily consumed (on the preceding day) by a corresponding rat in the test group, but were allowed water ad libitum. Rat Bcm4 was thus paired with Btm3, Bcf6 with Btm5 and Bcf8 with Btf9. Another "restricted food" control, paired with Btm1, died within a month and was excluded from the experiment. All the rats were kept in individual cages, excepting the 2 normal controls, which were caged together. The cages had wire net bottoms, allowing the feces and urine to drop through.

Each rat was weighed daily before feeding, at the same hour as nearly as possible. The food used was the McCollum stock diet I (casein 15 per cent, whole milk powder 10, unsalted butter 5.2, whole ground wheat

67.5, sodium chloride 0.8, calcium carbonate 1.5). The food was weighed and the daily consumption was recorded for each rat (but only from the 18th week of the experiment in series A). This food yields about 4 calories per gram, or about 3.5 calories on the basis of digestibility. Richter estimates 3.73 calories per gram for food of similar composition.

Each cage was provided with a water tube graduated to 100 cc., as used by Richter (1926). The daily consumption of water for each rat was thus easily determined. Ordinary tap water was used. It was found that the average evaporation per week from each water tube was about 5 cc.

TABLE 1

Age, body weight, nose-anus length and tail length of the rats used

RAT	AGE		BODY WEIGHT		NOSE-ANUS LENGTH	TAIL LENGTH
	Initial	Final	Initial	Final	Final	Final
Normal controls						
	days	*days*	*grams*	*grams*	*mm.*	*mm.*
Acf1	47	271	76	211	196	151
Acf2	47	272	83	211	203	153
Test rat held at constant body weight						
Atf1	47	271	74	75	149	126
Atf2	47	272	75	75	145	112
Atf3	47	273	65	66	128	100
Btm1	25	156	66	66	152	125
Btm3	25	161	70	70	155	127
Btm5	25	163	68	68	157	103
Btf9	23	147	49	53	145	116
"Restricted food" controls						
Bcm4	25	158	67	89	168	(stub)
Bcf6	23	159	42	83	162	134
Bcf8	23	138	41	78	152	130

This amount was therefore subtracted from the average apparent water consumption of the control rats. The thirsty test rats consumed their daily allowance of water usually within a minute, so that in this case the loss by evaporation was negligible. By drying in an oven at 100°C., the food also was found to contain about 22 per cent of water, which should be added to obtain the exact total amount of water actually consumed. This correction, however, has not been made in the records presented, and was likewise omitted by Richter.

The temperature in the animal room during the experiments was fairly constant at an average of about 24°C. (range 18 to 27°). The relative

humidity also was measured daily with a Tycos hygrometer, and averaged about 40 per cent (range 34 to 53 per cent).

At the close of the experiments, the animals were killed by ether inhalation and were autopsied. The various organs were then weighed and their water content was determined by drying in an electric oven at a temperature of 98 to 100°C.

Normal control rats. These two sister rats, Acf1 and Acf2, remained

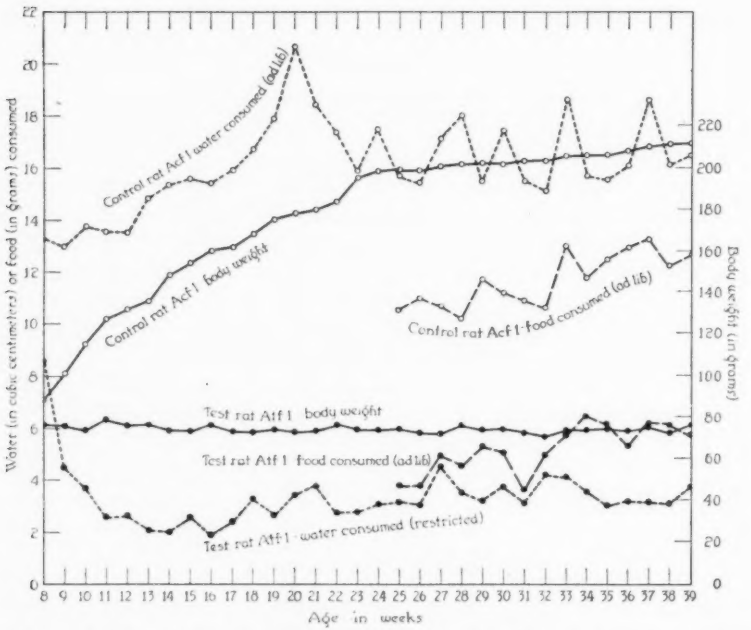
Water (in cubic centimeters) or food (in grams) consumed
Body weight (in grams)
Age in weeks
Control rat Acf1 water consumed (ad lib)
Control rat Acf1 body weight
Control rat Acf1 food consumed (ad lib)
Test rat Atf1 body weight
Test rat Atf1 food consumed (ad lib)
Test rat Atf1 water consumed (restricted)

Fig. 1. Graphs showing the body weight, water-intake and food consumption for the test rat Atf1 and the corresponding normal control rat Acf1. The normal control received food and water ad libitum. The test rats received food ad libitum, but the water was restricted to that necessary to maintain a constant body weight. The readings in each case represent the daily averages for the preceding week.

active and apparently healthy throughout the experiment, beginning at 47 days and extending to about 270 days of age. According to Donaldson's norm their final body weights (table 1) are about normal for body length though slightly subnormal for age. The tails are relatively short. The growth curve for Acf1, shown in figure 1, is normal. It also represents closely the growth for Acf2.

The food consumption for Acf1, recorded only from the age of 24 weeks

onward, evidently runs roughly parallel with the growth curve, as shown in figure 1. (The food and water for Acf1 are estimated as half the consumption for the two normal controls, caged together.) The water consumption increased from an average of about 13 cc. daily at the beginning to 16 or 18 cc. toward the end. There was an accountable rise to over 20 cc. daily at the 20th week of age.

Our figures for normal water consumption, even when corrected by adding the (22 per cent) water content of the ingested food, are somewhat

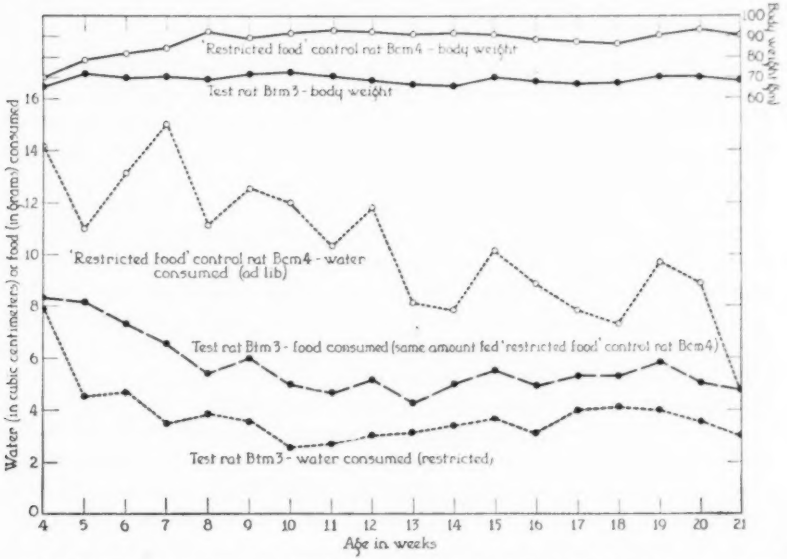
'Restricted food' control rat Bcm4 - body weight
Test rat Btm3 - body weight
'Restricted food' control rat Bcm4 - water consumed (ad lib)
Test rat Btm3 - food consumed (same amount fed 'restricted food' control rat Bcm4)
Test rat Btm3 - water consumed (restricted)
Water (in cubic centimeters) or food (in grams) consumed
Body weight (gms)
Age in weeks

Fig. 2. Graphs representing the body weight, water-intake and food consumption for the test rat Btm3 and the corresponding "restricted food" control Bcm4. The test rat received food ad libitum, but the water was restricted to that necessary to maintain a constant body weight. The "restricted food" control received water ad libitum, but the food was restricted to that voluntarily consumed by the corresponding test rat. The readings in each case represent the daily averages for the corresponding week.

below those reported by Richter (1926) and Richter and Brailey (1929). This discrepancy is hardly accounted for by our evaporation control (which averaged less than 1 cc. daily), or by differences in room temperature. There are evidently marked individual differences in the water-intake of normal rats of the same sex, age and weight. As noted by Richter and Brailey, the body weight increases more rapidly than the water consumption; this is true especially during the more active period of growth. There is also a general correspondence between the water con-

sumption and the food-intake, as was observed by Kleitmann (1927) in dogs. At autopsy, the various organs in this group appeared normal.

Test rats. The 7 test rats, held at nearly constant body weight by restricted water-intake, also remained active and in general apparently healthy, though becoming more or less emaciated. Their measurements are given in table 1. The 3 test rats of series A were older (47 days) when the experiment began, and were kept at nearly constant weight (65 to 75 grams) for a longer period of about 225 days. Nevertheless, their final weights were but slightly below Donaldson's norm for the corresponding nose-anus lengths. The 4 test rats of series B were younger (23 to 25 days) when the experiment began, and were kept at a slightly lower body weight for a shorter period of 124 to 138 days. They became relatively slenderer. In both series the tail length of the test rats was in most cases nearly normal for corresponding body length, and not elongated as found by Kudo (1921).

The degree to which the water consumption had to be restricted in order to prevent increase in weight in the test group is shown, and also for a representative rat in each series by the graphs in figure 1 (rat Atf1) and figure 2 (rat Btm3). Tables showing the complete data are omitted for economy of space. It is noteworthy that the amount of water required for maintenance of body weight under these conditions decreased rather rapidly from about 8 cc. daily average in the first week to a minimum of less than 3 cc. daily in the second or third month of the test. Thereafter the amount rose slowly, but in general remained below 4 cc. daily up to the end of the experiment The progressive decrease in water requirement is in general agreement with the results of Kudo (1921). He, however, did not note any final increase, probably because his experiments were for a shorter period of time.

The food voluntarily taken when the water was restricted likewise decreased progressively, as shown in figure 2. Although the food consumption ran roughly parallel with the water-intake, the food decreased more slowly from an average of about 8 grams daily in the first week to about 4 grams in the second or third month, with a slight increase later (also found in series A, as shown in fig. 1).

A progressive decrease in the amount of food required for the maintenance of body weight in young rats was likewise noted by Jackson (1915), and probably corresponds to the decrease in basal metabolism noted by various observers during prolonged inanition. It is evident that under such circumstances the animal organism in some way becomes more economical in the utilization of both food and water, but the exact mechanism of this phenomenon remains undetermined. In the earlier stages of inanition, both energy and water are made available by oxidation of the stored

body fat, but the amounts thus released would probably tend to decrease progressively from the outset.

The appearances of the body and organs of the test rats in general resembled those observed by Kudo (1921) in somewhat smaller rats on shorter experiments. The changes in organ weights were also generally similar to those noted by Kudo, excepting that in our series the liver was reduced in size and the gonads nearly normal in weight. Somewhat comparable dystrophic growth changes during different types of inanition in various species are reviewed by Jackson (1925, 1929).

The water content of the various organs was compared with Lowrey's figures (cf. Donaldson, 1924) for normal organs of corresponding weight, by making the necessary interpolations. While it appears unnecessary to review the data in detail, they indicate that all of the organs in our test rats had become more or less dehydrated. The organs in these rats averaged lower in percentages of water content approximately as follows: skin −8.0; spleen −4.7; muscle −2.7; eyeballs −2.5; lungs −2.4; heart −2.1; liver −1.9; kidneys −1.7; testes −1.1; brain −1.1.

"Restricted food" controls. These 3 rats (Bcm4, Bcf6, Bcf8) likewise remained healthy and active. They became somewhat less emaciated than the corresponding test rats, though still quite subnormal in weight according to length (table 1). Their tail length was nearly normal. Data for the changes in body weight, water-intake and food consumption of this group are given in table 1 and for a representative rat (Bcm4) in figure 2. With the same food allowance as was consumed by the corresponding test rats, but with unrestricted water-intake, this group made notable gains in weight. Rat Bcm4 increased from 67 grams to 89 grams, a gain of 33 per cent; Bcf6 from 42 to 83 grams, a gain of 98 per cent; and Bcf8 from 41 to 78 grams, a gain of 90 per cent in weight. Thus the representative shown in figure 2 (Bcm4) made by far the smallest gain of the 3 rats in this group. It is clear that this growth in comparison with the stationary weight of the corresponding test rats, was due solely to the increased water-intake, since the food and all other environmental factors were the same in both groups. (The slight amount of salts in the additional tap water may probably be regarded as insignificant.)

The water consumption that enabled this group of rats to make such remarkable gains is shown for one representative (Bcm4) in figure 2. In all cases the voluntary water-intake of the "restricted food" group was 2 to 3 times as great as that of the test rats, both having the same food consumption. In both groups the water-intake is roughly parallel with the food consumption, but in the "restricted food" controls the decrease in the water intake appears more progressive throughout the experiment, with less tendency to reach a minimum and then increase again (fig. 2).

At autopsy, the "restricted food" controls in general appeared less emaciated and in better condition than the corresponding test rats, but still showed certain abnormalities. The abdominal viscera, especially the gastro-intestinal tract, appeared somewhat less atrophic, but the abdominal and subcutaneous fat was very scanty. The urinary bladder was empty.

The fresh organ weights in this group differed considerably from Donaldson's norm for corresponding body weight and also from those in the corresponding test group. In the test rats the eyeballs, kidneys, skeleton, ovaries, testes, suprarenals, and hypophysis averaged definitely above the norm for weight; the liver, spleen, skin, thymus and thyroid averaged markedly below the norm; while the brain, heart, lungs, stomach, submaxillary glands and muscles averaged close to the norm (within 10 per cent). In the "restricted food" controls the eyeballs, ovaries, testes, submaxillary glands and muscles averaged definitely above the norm; the liver, spleen, skin, thymus, thyroid, lungs and suprarenals below the norm; while the brain, heart, stomach, kidneys, skeleton and hypophysis averaged close to the norm (within 10 per cent).

In percentage water content, the organs of the "restricted food" controls, excepting the liver and muscles, appeared somewhat dehydrated. The differences (in comparison with the norm) were approximately as follows: skin −9.5; spleen −2.8; muscles + 0.1; eyeballs −2.6; lungs −3.3; heart −0.3; liver +0.2; kidneys −2.4; testes −0.7; brain −0.2. In most organs the dehydration was less marked than in the corresponding test group (above mentioned).

Again it may be noted that (aside from accidental individual variations) the differences between the test rats and the "restricted food" controls, in organ weights and water content as well as in body weight, must be ascribed to the difference in water-intake, since essentially all other factors were held constant. The importance of the water-intake as an independent factor in growth is thus clearly demonstrated. Restriction of water-intake therefore interferes with growth on its own account, as well as through the associated decrease in the consumption of food.

SUMMARY

1. Young rats were held at nearly constant body weight for several months by a restricted water-intake, allowing food ad libitum. These test rats showed a progressively decreasing requirement for water up to the second or third month of the experiment, after which there was a tendency toward a slightly increasing requirement. The food consumption was roughly parallel with the water-intake.

2. These test rats became emaciated, with changes in appearance and in organ weights resembling those previously found by Kudo in similar but shorter tests. The organs also become variably dehydrated.

3. "Restricted food" controls were given (with water ad libitum) the same amount of food as was voluntarily consumed by the corresponding test rats. These control rats made a remarkable gain in body weight, ranging from 33 to 98 per cent. This gain was due solely to the increased water-intake, since the other factors were held constant.

4. These "restricted food" controls also became emaciated, but the changes, including those in organ weights and dehydration, were in general somewhat less than were found in the corresponding test rats. These differences likewise must be ascribed to the increased water-intake. The importance of the water-intake in growth, independent of the food consumption, is thus demonstrated.

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AN ANALYSIS OF THE LOW-VOLTAGE ELEMENTS OF THE ACTION-POTENTIAL WAVE IN NERVE

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A number of recent investigations of the form of the action potential wave in nerve (Levin, 1927; Davis, Pascual and Rice, 1928; Amberson and Downing, 1929; Gerard, 1930; Gasser and Erlanger, 1930) have been directed to the study of certain prolonged low-voltage elements of the wave which follow the more obvious and better known high-voltage phase, whose duration does not exceed a few thousandths of a second. The present study represents a further development of the method by which Amberson and Downing (1929) were able, in a roughly qualitative manner, to detect the presence of, and follow some of the major changes in, these low-voltage elements. Their conclusions rested, in all cases, upon a comparison of the form of galvanometer deflection given by the action-potential wave with that given by a physical control curve, produced by a break induction shock. Such a comparison showed the presence, in the single action-potential wave, of a low-voltage negative phase, immediately succeeding the high voltage phase, followed, after 0.5 to 1 second by an "after-positivity." Amberson and Downing attempted to analyze their curves by an adaptation of an arithmetic method of curve analysis, often used in the analysis of tide and sound waves, and applied to their myothermic studies by Hill and Hartree (1920), but were not able to secure trustworthy solutions. Amberson (1930) has pointed out some of the difficulties inherent in this method of curve analysis. In the case of records of action-potential waves the greatest difficulty in securing a trustworthy arithmetic solution arises from the fact that a very brief high-voltage phase is immediately succeeded by a much more prolonged low-voltage phase of a different order of magnitude, so that it is impossible clearly to distinguish the one phase from the other in galvanometer records whose time to maximum deflection ranges between 0.3 and 0.7 second.

We have, therefore, developed another type of analysis which gives a much more direct approach to the determination of the magnitude and duration of the low-voltage elements. For technical reasons we have not

found it practicable to study the "after-positivity" which regularly appears toward the end of the single action-potential wave. We have, however, succeeded in developing a method which gives semi-quantitative information concerning the duration and magnitude of the low-voltage negative phase which immediately follows the high voltage phase. This report will describe this method and will present the results of a number of studies on the form and magnitude of both high and low-voltage phases of the action-potential wave, under various physical and physiological conditions. We also include some results secured with photographic recording, where these confirm our direct analysis, particularly with reference to the behavior of the low-voltage elements during oxygen-lack.

The best terminology to be employed in connection with these phenomena is still a matter of dispute among those interested in such studies. The low-voltage elements have been variously spoken of as "retention" (Levin, 1927), "persistent negativity" and "after-positivity" (Amberson and Downing, 1929), "delayed potential" (Gerard, 1930), and "after-potential" (Gasser and Erlanger, 1930). The latter workers propose that the initial high-voltage phase be referred to as the "spike." We have ourselves come to speak of the two phases of the action-potential wave as "high-voltage" and "low-voltage," respectively. We have definitely abandoned the terms "retention" and "persistent negativity" since they connote the continuation of a first process rather than the inauguration of a second, and believe that the phrase "delayed-potential" may give a false impression of some mechanism which has been retarded in its speed, whereas it merely follows another process in time, and is not delayed in any true sense. The terms "spike" and "after-potential," proposed by Gasser and Erlanger (1930) are quite non-committal and suggest a difference in duration, yet may fail, we believe, to suggest the second great difference between the two phases, namely, the voltage. We believe, therefore, that some virtue resides in our own very general description of the two elements of the wave, as "high-voltage" and "low-voltage" phases, and will continue so to speak, adopting, however, the terminology of Gasser and Erlanger as convenient synonyms, and occasionally combining the two modes of speech where clarity of thought appears to be aided by such a procedure.

We might also point out that we have approached these studies from a somewhat different point of view from that held by Gasser and Erlanger in their recent paper. The striking influence of previous activity upon the duration and magnitude of the low-voltage elements, described below in the first part of the experimental section, has convinced us that, in order to simulate the conditions present in normal nerve within the body, we must maintain an active state by periods of repetitive stimulation, preceding each reading taken on the single action-potential wave. Gasser and Erlanger speak of this state as fatigue, and believe that it does not repre-

sent the normal state of the tissue, stressing rather the first records obtained from fresh nerve as giving the truer picture of bodily conditions. Further study must be made before it will be possible to decide finally between these two viewpoints. The difference is one of interpretation, not of fact. It can at least be said, for our own strategy of experimentation, that we secure the maximum opportunity to detect and dissect the low-voltage after-potential, and to follow its changes when experimentally modified.

METHODS. The method of analysis which we have devised is essentially an application of the principle by which Bernstein (1868), long before the development of rapid recording instruments, succeeded in determining the approximate form and time relations of the high-voltage phase in nerve. Our method depends upon the use of a large pendulum, with a period of one second, which, in its movement, opens one, two or three keys. Two of these keys are movable and may be set to various distances from the one fixed key. The time required for the pendulum to move from one key to another is accurately known. In the direct analysis of the single action-potential wave the opening of the first key generates a break induction shock which stimulates the nerve; the opening of the second key breaks the galvanometer circuit. As a result the galvanometer receives only that portion of the whole electrical discharge which is generated within the period between the opening of the two keys. Obviously the opening of the second key, breaking the galvanometer circuit, will cause the latter to return to its physical zero, at the conclusion of its movement. If this zero is different from the previous position of the galvanometer at the moment of stimulation, an error in the reading of the maximal deflection will arise. In order to secure a true measure of the electrical discharge, up to any given time, it is therefore necessary to adjust the galvanometer so that, at the moment when the reading is taken, the moving system is at its physical zero, even though the circuit has not been broken This is most conveniently done by the use of a Leeds and Northrup Type K potentiometer, introduced to balance off the injury potential of the nerve. Frequent checks are made of the position of the physical zero, which may shift slightly with time. When the final adjustment has been made the pendulum is allowed to swing, and the maximal deflection is read by direct observation of the reflected beam against a translucent scale. At the end of the galvanometer movement the moving system returns to the point from which it started. A true measure of the electrical discharge is thus secured.

When two waves, following each other at a brief interval, are to be studied, three keys are used. The first two, set at a known time interval, generate two break induction shocks which stimulate the nerve; the opening of the third key breaks the galvanometer circuit.

In carrying through the analyses we first determine the maximal deflection given by the whole action-potential wave. This value includes, of course, both high- and low-voltage phases. We then determine the maximal deflection given by the potential which developes within 10σ after the stimulus. This value is a measure of the high-voltage phase in the main, although some element of the negative after-potential is also included, as the tables and figures of the experimental section will indicate. We then read, in succession, the maximal deflections produced by those portions of the wave which appear up to 60, 110, 160, 210, 260, 310 and 360σ, or, in many cases, take readings at only a few of these stations. It is then possible to determine, by differences between successive readings, the amount of electricity which has discharged within successive periods of times. These values by difference are shown in the figures below, and determine the average magnitude of the potential within the successive blocks of the analysis. A few analyses of high-voltage phase in 1 and 2σ blocks have been made; no great accuracy is possible in such a determination.

The accuracy of readings taken in this manner absolutely depends upon the stability of the physical zero. We operate at sensitivities of between 60 and 80 cm. per millivolt. In spite of this high sensitivity we have been fortunate in securing a physical zero which is usually constant to 1 mm. for several minutes, and almost always is good to 2 mm. for the same period. For this advantage we are deeply indebted to Mr. Downing, who built the moving system, and adjusted it to be astatic in our own laboratory, in the manner described by Amberson and Downing (1929).

The consistency of our readings can best be judged by an inspection of the tables in the experimental section, in which the values for several experiments are given.

Limitations of the method. Certain physical limitations exist in this method of direct analysis, which make it impossible to arrive at absolute values for the effects under investigation. These limitations arise in part from the fact that the action-potential wave in nerve contains low-voltage elements which often endure for much more than the ballistic period of the galvanometer. Under such circumstances the maximal deflection is not a true measure of the total amount of electricity which has passed, but indicates less than the true amount. In some of our readings this error is undoubtedly considerable, and may affect the value for the whole wave by as much as 25 per cent. The error is in such a direction, however, that any conclusions which we reach concerning the magnitude and duration of the low-voltage elements are conservative.

Throughout the work here reported the galvanometer has been set to one constant period and sensitivity. The period within which it will behave ballistically has been determined by calibration with two break induction shocks, at various intervals. Between 0 and 200σ the summation of

potential is complete. At 300σ the galvanometer is just beyond its ballistic period, giving a deflection which is only 96 per cent of what should be obtained if summation were absolute. At 360σ the deflection is reduced to 89 per cent of what should be given. Beyond this time we cannot read with our pendulum; the error must become progressively greater, so that the terminal portions of the negative after-potential cannot be determined with any accuracy.

The first portions of the analysis, however, in which the electrical discharges fall within the ballistic period of the galvanometer, are not complicated by this factor. If there were no other complicating factor the increments of deflection secured, as longer and longer portions of the action-potential wave are allowed to flow into the galvanometer, should be strictly comparable up to 260σ. A further complication, however, is present, which operates to distort the readings in a direction which can be empirically determined, but which cannot accurately be allowed for.

TABLE 1

Calibration values for experiment of May 29, 1930

DURATION OF E.M.F.	MAGNITUDE OF E.M.F.	MAXIMAL DEFLECTION	CALCULATED DEFLECTION PER MILLIVOLT PER 50σ
σ	*m. v.*	*mm.*	*mm.*
2	30	32	27
6	30	112	31
10	10	64	32
50	3	117	39
100	1	91	45

We discovered this complication when we arranged to let a potentiometer, set at various known voltages, discharge into the galvanometer circuit for various known brief periods of time, expecting thereby to secure a set of calibration readings by which our unknown values could be evaluated. In all cases we have found that, for the same voltage, the maximal deflection of the galvanometer increases more than in direct proportion to the time of discharge, as the latter is lengthened, even though we work entirely within the known ballistic period of the galvanometer.

The magnitude of this discrepancy, whose source we have not been able to locate, is not great enough to make any essential change in the results which we have to present, but its presence means that our readings are not absolute quantities. The magnitude of the effect may be seen in table 1, which presents the calibration data from a typical experiment. After the suppression of all action-potentials in the nerve by the application of ether vapor these values were secured by impressing known E. M. F.s for known periods upon the whole circuit, including the nerve in

place upon the lead-off electrodes. From the last column it will be seen that the deflection calculated for 1 millivolt enduring for 50σ rises steadily as the duration of the potential increases, becoming almost 50 per cent greater when the duration is 100σ than when it is 10σ only. For the evaluation of the analysis data in the following experimental sections we have arbitrarily accepted the values secured when the duration of the impressed E. M. F. is 50σ. These values approximately represent an average between the readings secured for durations 10σ and 100σ. Their use probably means that the voltages assigned to the first 10σ blocks of the analyses are too low by about 15 per cent, while those assigned to blocks beyond 50σ are too great by about the same amount.

Throughout the present study we have used the sciatic nerves of large bull frogs, and unless otherwise noted have worked at 20°C. The nerve chamber and general procedure are identical with that described by Amberson and Downing (1929).

RESULTS. A. *Rest and activity.* The intimate relation between the state of activity of the nerve and the magnitude and duration of the low-voltage elements of the single action-potential wave, described by Amberson and Downing (1929) from photographic records, has been even more strikingly evident in the results secured by direct analysis. Indeed the relationship is so important that we find it necessary rigidly to control this factor in all studies in which the effects of other influences are being studied. We routinely excite the nerve for a period of half a minute, with repetitive stimuli at a rate of approximately 60 per second, before the application of the one or two stimuli which are used to give the wave or waves for direct analysis. These latter stimuli are applied within the two-minute period immediately succeeding the end of the rapid repetitive stimulation. Under such conditions the low-voltage elements of the action-potential wave are developed to their maximum. This effect is very marked in mammalian nerve (Amberson, 1929) in which the disappearance of the low-voltage elements after cessation of activity is much more rapid than in frog nerve. When single waves are taken at longer and longer times after the repetitive stimulation the low-voltage elements gradually diminish, and may almost or quite disappear when the period of inactivity exceeds one hour in duration.

The nature of this effect, as disclosed by direct analysis, is shown graphically in figure 1. The readings which were secured in this particular experiment are given in table 2. Our apparatus, designed to study the much more prolonged low-voltage elements, does not permit any great accuracy in the analysis of the relatively brief high-voltage phase. Rough analyses of this phase may, however, be made by our method, and in a few experiments we have secured such readings. The data of table 2 and figure 1 show the results of such an analysis of the high-voltage phase, as well

as of the low-voltage elements which succeed it. The relative magnitude of the two phases will be clear from an inspection of charts 1 and 2 of figure 1. These show the first portions of the two analyses only. In charts 3 and 4 of the same figure the complete data are plotted to different time and voltage scales, so as to magnify the size and show the duration of the low-voltage after-potential. The first block of each analysis in these latter charts and in all other figures below is shown as occurring between 2 and 10σ, allowing 2σ for conduction time. The height of the block represents average voltage over this 8σ period; the actual peak voltage of the "spike" is, of course, much higher, and is more nearly approximated in charts 1 and 2 of figure 1 than in any other charts given in this paper, in which no further attempts will be made to show the finer detail of the high-voltage phase, which has, indeed, usually not been studied.

TABLE 2

Analysis of the single action potential wave before and after activity

1. BEFORE ACTIVITY		2. AFTER ACTIVITY	
Galvanometer circuit closed for:	Maximal deflection	Galvanometer circuit closed for:	Maximal deflection
σ	*mm.*	σ	*mm.*
2	0, 0	2	0
2.5	0	4	4.0, 4.0
3	0.5, 1.0	6	6.0, 6.0
4	4.5, 4.5	8	8.5, 9.0
6	6.5, 6.0	10	9.5, 9.5
8	7.0, 7.0	60	26, 25.5
10	8.0, 8.0	160	45, 45
25	9.5, 9.0	260	58, 58
35	10, 10	360	66, 68
Whole wave	10, 10	Whole wave	78, 79

The last block of the analysis, shown in stipple, differs from all others in that the deflection assigned to it represents the difference between that given by the whole wave, and that obtained when the galvanometer circuit is opened after 360σ. The actual magnitude and duration of this terminal portion of the negative after-potential cannot be accurately determined by our present apparatus. In most cases we have, therefore, arbitrarily plotted this final value as occurring between 360 and 500σ.

From the data of this experiment it will be clear that the effect of previous activity is to cause the development of a very prominent negative after-potential. In the inactive nerve the single action-potential wave generated by a maximal break induction shock, consists in the main of the high-voltage phase, 80 per cent of the total electrical discharge occurring within 10σ of the stimulus, while all of the wave is over within 35σ. Upon

activation by successive periods of repetitive stimulation the maximal deflection given by the whole wave, evoked by the same strength of stimulus, rises to 79 mm., or nearly eight times that previously given. Nearly all of this increase is found to occur after 10σ, so that the first portion of the wave, only slightly affected by the activity, now constitutes less than

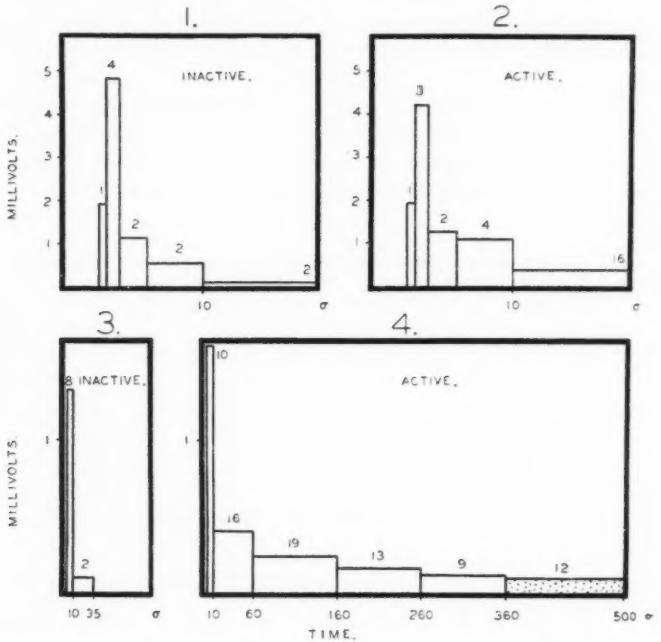

Fig. 1. Analyses of the single action potential wave before and after activity. The original readings are given in table 3. In charts 1 and 2 the first portions only are given. Analyses of the high voltage phase are here shown. In charts 3 and 4 the full analyses are given. The numbers over each block represent the millimeters of deflection allotted to each, as determined by differences between successive readings in the analysis. The high voltage phase is here shown as a block between 2 and 10σ. The calibration data for this experiment are given in table 2. Calibration value used for whole figure: 1 millivolt for 50σ gives 39 mm. deflection.

15 per cent of the total electrical discharge. This and other analyses of the high-voltage phase indicate that this phase is little, if at all, affected by a number of factors which may radically alter the character of the low-voltage elements. In the data of table 2 it will be seen that the two analyses are identical up to 6σ, within the error of the method. Beyond 6σ the two sets of readings diverge, and are significantly different at 10σ,

showing that the effect of previous activity is evident very early in the action-potential wave.

The attainment of a steady state upon activation by repetitive stimuli is by no means immediate. In this experiment the rise in the maximal deflection given by the single wave, induced by the previous activity, did not reach its final maximum until after eight half-minute periods of repetitive stimulation. Before activation the whole wave, shown in analysis 1 of table 2 and in charts 1 and 3 of figure 1, gave 10 mm. deflection. Then, in succession, after each period of multiple stimulation, the whole wave gave 25, 32, 45, 51, 64, 71, 74, and 79 mm. The last value appeared to represent the maximum development of the low-voltage phase possible under these conditions, since further stimulation made no further change in the value. Analysis 2 of table 2, shown graphically in charts 2 and 4 of figure 1 was then secured.

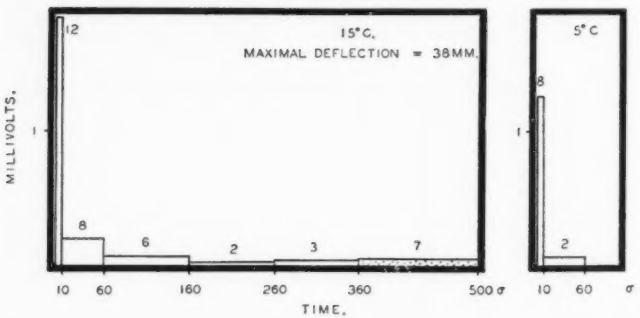

Fig. 2. Effect of temperature upon the single action potential wave. The analysis at 5°C. was carried out thirty minutes after the change from 15°C. Calibration value: 1 millivolt for 50σ gives 39 mm. deflection.

B. *Effect of temperature.* The low-voltage elements of the action-potential wave in frog nerve reach their maximum development at temperatures between 15° and 20°C. Above and below this range they are reduced in magnitude. The reduction at higher temperatures, however, is relatively slight compared with the almost complete suppression of this phase of the wave at temperatures of 5°C. or below. Within a few minutes a change of temperature from 15° to 5° may reduce a powerfully developed negative after-potential to the merest remnant, while effecting little change in the amount of electricity associated with the high-voltage phase, although the distribution of the latter in time is considerably altered.

The analyses of figure 2 will indicate the nature of this effect. In all cases the readings are taken very shortly (within 2 minutes) after a previous repetitive stimulation enduring for 30 seconds; a steady state is thus

insured. At 15°C. the electrical discharge observed in the first 10σ gives 12 mm. of deflection, out of a total of 37 to 40 for the whole wave; the negative after-potential endures for much longer than 360σ. At 5°C. 8 out of a total of 10 mm. is given within the first 10σ. At this temperature the negative after-potential is very much reduced in magnitude and duration, and appears to be completely discharged within 60σ. These observations upon the influence of low temperature in suppressing the negative after

TABLE 3

Photographic records of the single action potential wave taken before, during and after oxygen lack. May 9, 1930

	TIME TO MAXIMAL DEFLECTION	AVERAGE TIME	AVERAGE MAXIMAL DEFLECTION
	σ	σ	*mm.*
Physical control	450, 455, 451	452	45
Nerve			
In air	549, 517, 521	529	15
In nitrogen for:			
1 hour	598, 631	615	16
1 hour 30 minutes	630, 630	630	26
2 hours	541, 572, 548	554	15
2 hours 40 minutes	400, 434	417	5
3 hours 30 minutes	No deflection		0
In air again for:			
10 minutes	483, 494	488	28
40 minutes	662, 636	649	49
1 hour	687, 695	691	57

The table shows 1, that the time to maximal deflection of the galvanometer, given by the single action potential wave in fresh nerve is considerably longer than the physical control, indicating the presence of a negative after-potential; 2, that in nitrogen this after-potential is first increased, and then, after 2 hours suppressed, long before the disappearance of the high-voltage phase, which continues for 3 hours and 20 minutes, as shown by repetitive stimulation; 3, that, upon readmission of air the after-potential develops again, and becomes larger than at any previous time. The maximal deflection rises and falls as the after-potential appears and disappears. The records taken at 2 hours and 40 minutes in nitrogen have too small an amplitude to be read with great accuracy, but they clearly indicate that the after-potentials have disappeared.

potential are in agreement with the findings of Gasser and Erlanger (1930) extending their work to show that this suppression occurs even though the nerve is continually activated by periods of repetitive stimulation, before each reading.

C. *The effect of oxygen lack.* Before the development of the method of direct analysis a group of experiments with photographic recording of the whole wave had indicated that the low-voltage elements, while frequently

showing an augmentation shortly after the removal of oxygen, were then suppressed long before the disappearance of the high-voltage phase. It was observed that the form of galvanometer deflection given by the single action-potential wave, while at first differing radically from the physical control, came finally, in the absence of oxygen, to approximate it so closely that the curve was indistinguishable from it. In table 3 are given the results of one experiment; the magnitude of the displacement of the maxima of the curves given by single action-potential waves, when compared with

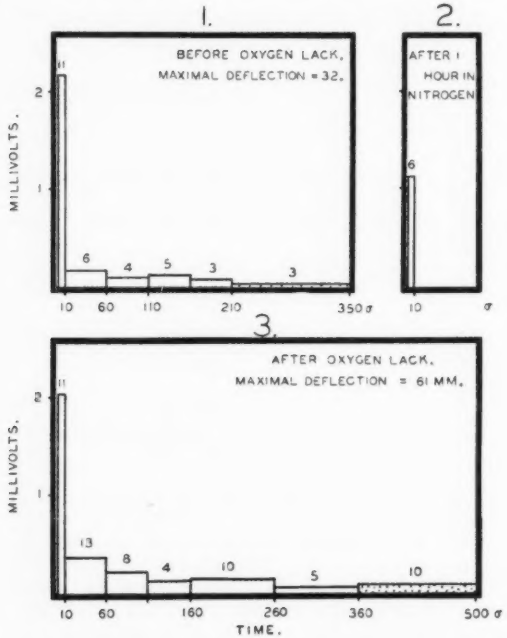

Fig. 3. Effect of oxygen lack upon the single action potential wave. Calibration value: 1 millivolt for 50σ gives 35 mm. deflection.

the control, at various times before, during, and after the removal of oxygen, are here shown. In this experiment the high-voltage phase persisted for more than an hour after the disappearance of the low-voltage phase. Upon the readmission of oxygen the photographic records showed a return of the low-voltage elements within a few minutes. In almost every case they became very powerfully developed, so that the temporal displacement of the maxima of the galvanometer movements rose to values higher than those obtained at any previous period. This was associated with a considerable increase in maximal deflection. These observations upon the

single action-potential wave are related to those reported by Gerard (1930a) for repetitive stimulation, in a manner which we will later discuss.

Upon the development of the present method of direct analysis we returned to examine these phenomena in the more precise way now possible. Direct analysis confirms the conclusions to which we had been led by an inspection of the photographic records. In figure 3 are given the results of three analyses of the single action-potential wave. In all cases the factor of previous activity has been controlled by half-minute periods of

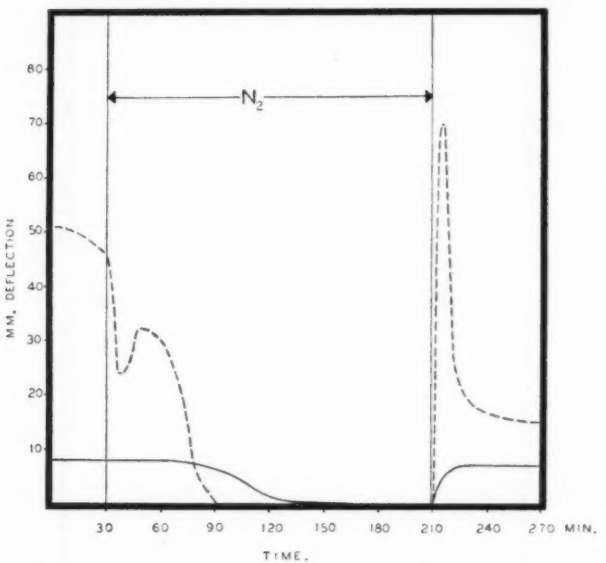

Fig. 4. Effect of oxygen lack upon the single action potential wave. The changes with time in the values for both the high- and low-voltage phases are shown. In nitrogen the low-voltage after-potential disappears more than an hour before the high-voltage phase; upon readmission of air it rises to a higher value than any previously observed.

———— Deflection produced by high voltage phase.

---------- Deflection produced by the rest of the wave.

repetitive stimulation shortly before each reading on the single wave. It is seen that in the fresh nerve a negative after-potential is present which is responsible for over 20 mm. of the maximal deflection of 32 mm. given by the whole wave. In nitrogen the after-potential, after some preliminary fluctuations, rapidly disappeared, so that an analysis carried out after one hour showed that the amount of electricity discharged within the first 10σ was practically identical with that given by the whole action-potential

wave, no difference greater than the experimental error of the readings being detectable. After the readmission of air the negative after-potential returned again within a few minutes, and became so powerfully developed that the whole wave rose to a value double that previously given by the wave in fresh nerve. This increase in maximal deflection is entirely due to an augmented electrical discharge after 10σ. The high-voltage phase, included within the first 10σ block of the analysis, shows no increase over

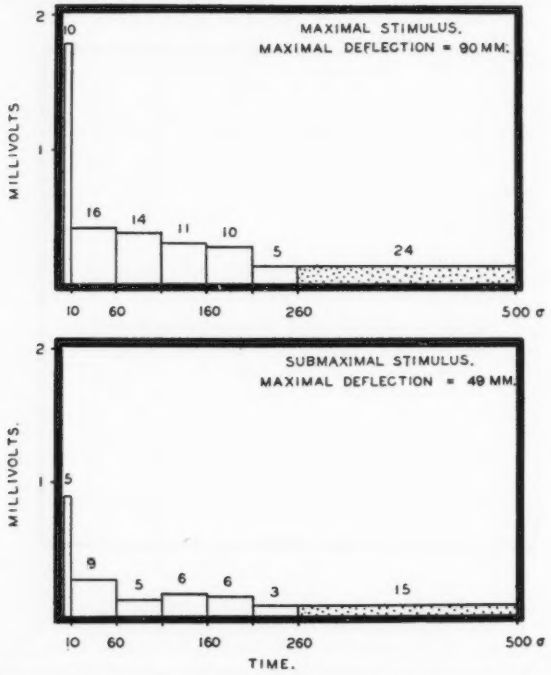

Fig. 5. Analyses to show that the after-potentials are present in both the more and the less excitable fibers. Calibration value: 1 millivolt for 50σ gives 35 mm. deflection.

that previously obtained in the fresh nerve; the readings secured after lack of oxygen are identical with those found before its removal within the error of the method. This phase behaves for us precisely as Heinbecker (1929) has found with the oscillograph.

The data from a third experiment are, in figure 4, presented in a different manner. The experiment is somewhat anomalous in that the post-asphyxial overshoot is relatively transient. The dissociation between the two phases of the action-potential wave, after one hour in nitrogen, is however

particularly clear; the after-potential disappearing completely after one hour, although the high-voltage phase persists for more than another hour thereafter.

We have taken advantage of the relatively large after-potentials which appear after a period of oxygen-lack to determine whether all fibers show the presence of these elements of the action-potential wave. In figure 5 are given two analyses of the single wave, as evoked by two stimuli of different strength. The one stimulus is slightly super-maximal, the second is of sufficient strength to give only about 50 per cent of the maximal deflection produced by the first. The two analyses, while differing in amplitude throughout, are entirely similar in form, the reduction in voltage being approximately constant throughout the whole wave, within the error of the method. It is evident that the after-potentials are as well developed in the more easily excited fibers as in those which are less easy to excite.

TABLE 4

Analysis of two action potential waves in air after a period of oxygen lack

GALVANOMETER CIRCUIT CLOSED FOR:	MAXIMAL DEFLECTION		
	Wave 1	Wave 2	Both together
σ	*mm.*	*mm.*	*mm.*
10	11.5, 10.5		
20	14, 14	11, 10.5	25, 23
70	26, 26	23, 24	45, 45
170	37, 36	36, 36	61, 62
270	46, 45	45, 47	74, 76
370	52, 52	51, 51	88, 91
Whole wave	61, 60	62, 61	101, 103

We have, however, some evidence that, in fresh nerve, the after-potentials are less prominent in the more excitable fibers.

A preliminary report of these observations on the influence of oxygen-lack was presented to the Physiological Society of Philadelphia. (Amberson, Parpart and Sanders, 1930.)

D. *Interference.* Direct analysis of the electric response associated with two action-potential waves, following each other at a short interval, has shown very clearly the nature of the interference between waves which Amberson and Downing (1929) inferred from their study of the maximal galvanometer deflections. We have used a stimulus interval of 10σ, which at 20°C. throws the second wave just beyond the relative refractory period of the first, and have carried through three analyses at once, taking data on the maximal deflections given by waves generated by each of two maximal stimuli, and by the two waves produced when both stimuli are used at the 10σ interval. Very clearly the two high-voltage phases come

through without diminution in the value of either, but the low-voltage elements are reduced in magnitude, in roughly the same amount over the

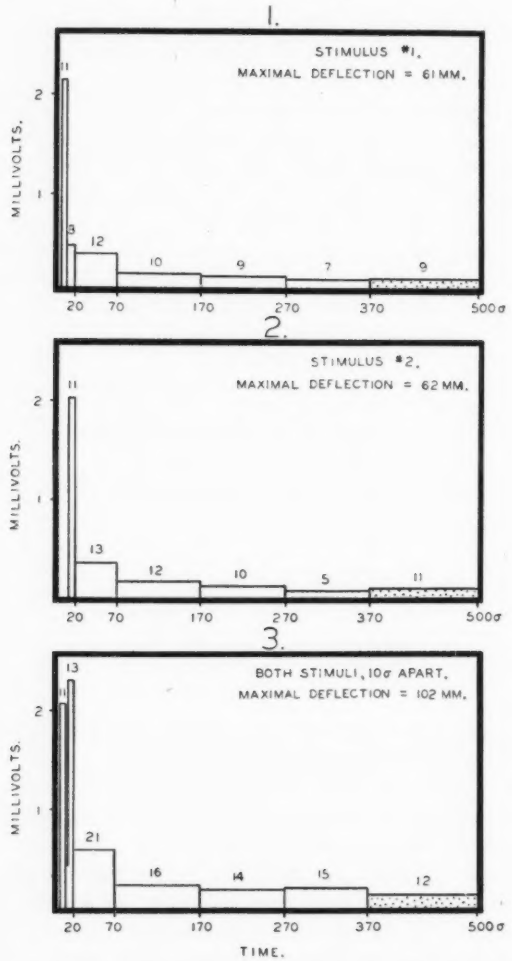

Fig. 6. Analyses of two waves from two different stimuli, taken separately, and together, at an interval of 10σ. Interference between the after-potentials of the two waves is shown. The original readings are given in table 5. Calibration value: 1 millivolt for 50σ gives 35 mm. deflection.

whole course. In figure 6 are shown three analyses done on one nerve during the three hours immediately after the readmission of air, following

a period of oxygen-lack. The original readings are given in table 4. These data are the most regular and consistent which we have secured, the value for the whole wave having remained constant for the whole period of the experiment in a truly remarkable manner.

The interference between the waves, in this instance, is of such a magnitude that the two waves together give, in their after-potentials, only about 80 per cent of that which would be expected if summation were complete. By analogy with the better understood subnormal responses of nerve in the classical relative refractory period, in which it is known that the high-voltage phase is subnormal in magnitude (Gotch, 1910; Forbes, Ray and Griffith, 1923; Gasser and Erlanger, 1925), we might interpret these results as pointing to a very prolonged relative refractory phase for the after-potential, in which this element of the second wave is very much reduced in amount. If we thus assign all of the observed reduction to the second wave we may conclude that its after-potential is reduced to about 60 per cent of that in nerve not previously excited for some seconds.

We have here presented data secured under conditions where the low-voltage elements are highly developed, so that large deflections are obtained, and the experimental error of reading is correspondingly reduced. We have, however, a number of other experiments done on fresh nerve, and on nerve in expired air. In all cases the results are similar to those here given. The high-voltage phases come through without interference, the low-voltage phases are reduced in amount, when two waves follow each other at a 10σ interval. The only difference worthy of mention is that, in expired air (low CO_2) the first portion of the low-voltage phase shows complete summation, the interference manifesting itself only in the later blocks of the analysis. The significance of this effect does not appear at present. It may also be mentioned that the action of CO_2, in exaggerating the whole electrical discharge, is mainly upon the after-potential.

Discussion. Much of the above data is confirmatory of the conclusions to which Amberson and Downing (1929) came, as a result of their photographic studies of the form of galvanometer movement. We are now in a position, however, to distinguish much more certainly between the high- and low-voltage phases of the action potential wave. These two phases appear to be the electrical signs of two separate processes, which may be experimentally dissociated from one another in a number of ways, by the suppression of the second while the first persists. The first process, whose electrical sign is the high-voltage "spike," is relatively anaerobic in nature, and shows little or no change in character with various changes in the physical and physiological conditions. The second process, whose electrical sign is the low-voltage after-potential, is relatively aerobic in nature, and is sensitively related to a number of factors, including O_2 and CO_2 tension, temperature and previous activity.

Of all of these relationships the effect of oxygen-lack would seem to be most significant. Our results are here in disagreement with the conclusions of several workers who have previously examined this point. Furusawa (1929) has extended the study of the electrical phenomena of the spider crab limb nerve, begun by Levin (1927), and reports that, when the nerve is in nitrogen, both action and injury potentials fall off together. After each stimulation the potential between injured and uninjured regions is further sharply reduced, as if, according to him, there were a permanent persistence of a very considerable negative after-potential, operating to oppose the injury potential. We feel that it is more logical to view this effect as a simple reduction of the injury potential rather than as an algebraic sum of two real but opposing electromotive forces. If we accept this alternative view we must conclude that the true after-potential is very much reduced, in nitrogen, since we must measure its duration by the period of time which elapses between the cessation of stimulation and the attainment of the new constant position of the galvanometer. Thus in figure 4, of Furusawa's paper, we may conclude that an after-potential which endures for 25 minutes in air is, in nitrogen, reduced to 6 minutes (or even to 2 minutes).

More recently Gerard (1930a) has studied the electrical response of frog nerve to repetitive stimulation during oxygen-lack, and concludes that the negative after-potential is progressively exaggerated up to the time of final loss of excitability. This conclusion rests upon a comparison of the maximal galvanometer deflections given by the action potentials obtained during repetitive stimulation, when the frequency of stimulation is altered. In the fresh nerve Gerard finds that when the stimulation rhythm increases from 88 to 304 per second, a ratio of 3.5, the maximal deflection always increases in some lower ratio. This discrepancy, he believes, arises largely from the fact that, at the higher frequencies, there is more interference between the negative after-potentials than at the lower frequencies, so that a larger percentage of each action potential wave is suppressed. A progressive fall of the $\frac{F}{S}$ ratio, during oxygen-lack, is recognized as being mainly due to a prolongation of absolute and relative refractory periods. The fall is, however, more marked in the region of the nerve within the nitrogen chamber than in a region beyond which remains in air. This greater fall of the ratio in the region from which oxygen is absent, taken in conjunction with a further fall immediately following the re-admission of air, leads Gerard to the conclusion that, in addition to changes in the refractory period, there is a progressive exaggeration of the after-potentials as asphyxia developes. The final return of the $\frac{F}{S}$ ratio to its original value, long after the re-admission of air, he interprets to mean a disappearance of the after-potentials.

At best such a criterion for the presence or absence of after-potentials is rather indirect. Even in fresh nerve Gerard finds that the $\frac{F}{S}$ ratio shows a considerable variation, from 2 to 3.5 at different leads from the same nerve at about the same time, so that even considerable changes in the ratio can hardly be regarded as significant. Although the value is almost always less than the known ratio of stimulation rhythms (3.5) we question whether this discrepancy between the two values is a clear indication of the presence of an after-potential. At the faster rhythm of 303 stimuli per second each action potential wave is following its predecessor in the early part of the relative refractory phase of the latter, so that the high voltage phase of each wave after the first will be subnormal in size (Gotch, 1910; Forbes, Ray and Griffith, 1923; Gasser and Erlanger, 1925). In the absence of any other line of evidence for the existence of after-potentials we might reasonably explain the value of the ratio as due to subnormal impulses at the higher stimulation rhythm. In a somewhat analogous situation Amberson and Downing (1929) were unable to relate the similar discrepancy observed between the electrical responses to one stimulus, and to two stimuli at short intervals, with the presence of an after-potential, until, by a different experimental approach, they had directly detected the latter.

While the determination of the $\frac{F}{S}$ ratio does not directly demonstrate the presence, or measure the magnitude, of low voltage elements, we have no doubt that its value, and changes therein, are in part due to fluctuations in the after-potential. In several situations it is clear that the value of this ratio is diminished when, from other lines of evidence, it is known that the after-potential is increased. Thus Gerard (1930a) has shown that such a change in the ratio occurs during the post-asphyxial overshoot of action-potential, which occurs for him with repetitive stimulation as for us in the present work with one and two stimuli. Necheles and Gerard (1930) have observed a fall in the ratio upon the application of CO_2, which has long been known to produce a large after-potential. During oxygen-lack, however, the intervention of other factors may, we believe, operate to prevent any such correlation, so that a fall in the ratio is not trustworthy evidence for an increased after-potential. In particular the prolongation of both absolute and relative refractory periods during oxygen-lack appears to us to be a sufficient cause for the change in the ratio in this direction.

We have searched for some evidence, in a photographic study of the form of the galvanometer movement following cessation of repetitive stimulation in nitrogen, that negative after-potentials become more prominent as asphyxia proceeds. Contrary to our expectation we have been

unable to detect even a trace of negative after-potential under any circumstances. When an "after-positivity" is present, its presence is discernible certainly within 150σ after the end of stimulation, as evidenced by a departure of the nerve action potential record from a physical control curve; within this period there is no evidence for a negative phase, the two curves being superposable. With the disappearance of the "after-positivity" after an hour or more in nitrogen, the action potential record becomes identical with the control, no after-potential of either sign being discernible. The time of disappearance of the "after-positivity," following a repetitive stimulation, coincides very closely with the time at which the negative after-potential also disappears, as shown by direct analysis of the single action potential wave.

These two effects, therefore, are very intimately related, and may be but two differing symbols of one fundamental process. This conclusion is strengthened by the fact that the two effects also behave similarly at low temperatures, both being suppressed. We are not able to state the conditions which determine the polarity of the after-potential. We are, however, unable to agree with the position of Gasser and Erlanger (1930) who believe the "after-positivity" to be an artifact, arising from the fact that a strictly monophasic response cannot be obtained. Recognizing the fact that such an effect must be present, for both high- and low-voltage phases, we still are sufficiently impressed by the long persistence of the positive phase, in both the single wave (Amberson and Downing, 1929) and after a tetanus (Gerard, 1930b), to feel convinced that it is a definite entity.

Our observations on the effect of oxygen lack are rather difficult to harmonize with Gerard's (1927a) findings that the two phases of the nerve heat are, in nitrogen, reduced together, no such dissociation of the two phases occurring as in muscle. Indeed the recovery heat appears to be reduced somewhat less rapidly than is the initial heat. It was to be expected that the two outstanding physical events in stimulated nerve, the production of heat, and of electrical potential, would be very intimately related to each other. Such a correlation was clearly suggested by the work of Gerard, Hill, and Zotterman (1927) on the influence of frequency of stimulation upon both effects. However, no post-asphyxial overshoot of nerve heat has been observed, such as occurs in the action potential. It seems possible to us that these two physical manifestations may represent two forms into which chemical energy may transmute itself, the amount of energy going into each form depending upon the circumstances, so that, while normally both appear together, one may, under exceptional conditions such as oxygen-lack, be preferred to the other. Thus an anaerobic recovery heat may continue after the after-potential has disappeared.

While the nature of the relationship between recovery heat in nerve and low-voltage elements of the action potential wave must remain in doubt there appears to be a definite correlation between the latter and the respiratory phenomena during and after oxygen lack. The observation of a post-asphyxial overshoot of after-potential, following its complete suppression in nitrogen, is in good agreement with the findings of Gerard (1927b) and of Fenn (1930) who have reported an anaerobic oxygen debt in nerve, with an accelerated rate of oxygen consumption appearing after readmission of air. Fenn also finds an increased CO_2 production. The nature of the chemical reactions involved is not clear. The work of Gerard and Meyerhof (1927) and Holmes and Gerard (1930) indicates that lactic acid is not involved, and that no close analogy with the chemistry of muscle can be drawn.

We may now consider certain more theoretical aspects of the present study. Levin (1927) suggested that the after-potentials arise from "the accumulation of some products in the nerve during activity, their presence being responsible for the negative potential of the retention of action current and their elimination for the disappearance of the negativity." Furusawa (1929) has accepted this interpretation, and stresses the necessity of oxidative processes for the removal of the metabolites. Gerard (1930a) has adopted a somewhat similar view, holding that, in the after-potential, "the amount of potential is an index of the recovery still to be completed." He believes that, in oxygen lack, a magnified after-potential appears which depends immediately upon a delay in the restoration of the membrane; this in turn rests upon the retardation of an oxidative recovery reaction. Gasser and Erlanger (1930), while believing that the after-potential is a direct indication of an incomplete recovery in the physical state of the plasma membrane, recognize that possibly "the membrane closes when the reaction products" (from a chemical breakdown in the spike) "are reduced below an assigned value."

In all of these views there is the recognition of the probability that the electrical manifestations which compose the low-voltage elements are connected with a chemical process of recovery. We are in general agreement with this point of view. We have, however, become convinced that the after-potentials are a direct index of the recovery process itself rather than of failure to recover. This view is similar to that which Hering (1884) long ago proposed as an explanation of the after-positivity which follows a tetanus. To him this phenomenon was the evidence for a process of assimilation or recovery, by which the nerve is built back to its original resting state. In a similar manner we conceive the negative after-potential of the single wave (and probably the later "after-positivity" as well) to be the electrical sign of a second process, restorative in nature, which is actively engaged, either in making new material available to the nerve,

or in removing or transforming material which has appeared in connection with the events of the high-voltage phase. The intensity of this restorative or recovery process is, we believe, to some degree indicated by the magnitude of the after-potential at every instant. The evidence here presented shows that the mere accumulation of metabolites from some chemical breakdown associated with the high-voltage phase is not in itself sufficient to produce the after-potentials. If this were true they should be exaggerated at low temperatures and in oxygen lack, in both of which states recovery must be more difficult. Rather must we conceive that it is the act of removal of these metabolites, or, as an alternative, the act of replacement of their precursor, that is responsible for the after-potentials observed.

Between these two alternative possibilities we are not able to distinguish at present. The marked exaggeration of the negative after-potential by CO_2 and the equally striking effect of veratrin in the same direction (Graham, 1930) are very difficult to conceive in terms of the hypothesis that the second process represents removal of metabolites, since both treatments have relatively little effect upon the high-voltage phase, and hence can hardly be supposed materially to affect the amount of its metabolites which must be removed. On the other hand it is not clear why, if the second process is concerned with the assimilation of new material, there should be so large a production of this substance in these two states.

The effects of low temperature, oxygen lack, and previous activity may, however, all be logically conceived in terms of the hypothesis that the second process is one of the assimilation of new material. Being oxidative in nature, this process will be suppressed by oxygen lack, and, having a high temperature coefficient, it will be practically inhibited at low temperatures. In both cases the low-voltage elements will disappear or be much reduced. In inactive nerve, where the stock of precursor substance is at its maximum, the magnitude of this restorative process is small, and the after-potential is correspondingly reduced. After a period of activity, and with the readmission of air after oxygen lack, the partial or complete exhaustion of this substance leads to an accelerated rate of resynthesis, with a corresponding increase in the after-potential. The ultimate suppression of conductivity, in cold or in oxygen-lack, is due to the final exhaustion of the precursor substance, whose replacement is impossible under these conditions. This substance may be considered as identical with the "oxidizing reserve" of Gerard (1927c) and the whole conception is in general agreement with the hypothesis then advanced by him. We have, however, no information as to the validity, in detail, of the hypothetical equations proposed by him.

The failure of the low-voltage elements completely to summate, when two waves follow each other at a short interval, points to a significant

characteristic of the recovery process. If magnitude of potential (or total quantity of electricity) can be considered as a direct measure of the amount of precursor substance synthesized, the recovery process appears to be unable to reform this substance at twice the speed possible in the single wave alone. With a rapid series of stimuli, therefore, the precursor material will be slowly reduced, reaching a new concentration level, where removal and resynthesis are again in balance. At the conclusion of such a stimulation the original level will be gradually restored, by a spontaneous, but slow, recovery process, whose electrical sign may be the long-continuing after-positivity. If, upon this relatively slow recovery, we superpose a single action potential wave, as in our experiments, we appear to furnish the opportunity for a momentarily greatly accelerated rate of resynthesis, with a correspondingly large after-potential. The high voltage phase, therefore, appears to supply a factor which initiates or accelerates the second reaction which leads to recovery.

It must be recognized that any such conception leaves many questions unanswered and must be held quite tentatively until we have a clearer picture of the physical and chemical events associated with the nerve impulse. Actually we measure, in these studies, only a certain type of physical change, presumably occurring at or in the plasma membrane, and it may well be asked whether any attempt should be made to go beyond or below the electrical phenomena actually observed. In justification for such attempts we may point out that the low-voltage elements of the action potential wave show so fluctuating a behavior, and are so easily affected by so many factors, that a chemical mechanism at the base of the effects would seem to be clearly indicated, since no purely physical system should show so labile a character.

The nature of the relationships between chemical changes in nerve and physical alterations of the membrane structure must remain problematical. It is entirely probable, however, that in both phases of the action potential wave the sequence of physical modifications in the membrane accompanies a series of chemical events. The electrical changes, therefore, are the expression of those events only by indirection, a physical modification of the membrane mediating the connection. There is some evidence (Amberson and Klein, 1928; Sands and Amberson, 1928) that the electromotive behavior of animal membranes is controlled by an ampholyte component, probably a protein, so that the magnitude of a membrane potential is intimately related to the pH of the tissue. Above an isoelectric point animal membranes are negatively charged, and appear to retard or inhibit the movement through them of ions of the same electrical sign. As the isoelectric point is approached the magnitude of the membrane potential diminishes as a result of a decrease in membrane charge, controlled by the dissociation state of the ampholyte; the potential is reversed

below the isoelectric point. The isoelectric point of many animal membranes is not far below the pH of the blood and tissues. It is entirely conceivable that the whole negative portion of the action potential wave is the physical expression of a diminution in pH, which throws the membrane closer to its isoelectric point, and so diminishes the potential across it. By analogy a positive phase might arise from an increase in pH. These effects might occur without any such changes in the concentration of the neutral salts as are visioned in the familiar theories of ionic stimulation, activity arising rather as the result of changes in H and OH ion concentration, induced by the stimulus, as Bethe (1916) has argued. The decline of the injury potential during oxygen-lack, and its recovery in air may similarly be conceived of as due to an accumulation, and later removal, of acid products.

The question as to whether or not the development of the low-voltage elements of the action potential wave is due to a state of fatigue deserves some final consideration. Gasser and Erlanger (1930) believe this to be the case. The doctrine of the non-fatiguability of nerve fibers might well be questioned in the light of such observations as that of a marked increase in the duration of the absolute refractory period with activity (Field and Brücke, 1926) and the lessened heat production, oxygen consumption, and action potential per impulse obtained when nerve is stimulated at a faster rate (Gerard, Hill, and Zottermann, 1927; Gerard 1927a). Gerard and Forbes (1927) point out that such changes may well arise, not as the result of fatigue, but from an adaptation or "equilibration" (Gerard, 1927b) of the tissue to a new state of activity. True fatigue phenomena would proceed toward final extinction of all activity. We are definitely in accord with this view, rather than with the conception that a true fatigue is responsible for the effects under discussion. When the factor of previous activity is uniformly controlled, and all other factors remain the same, the behavior of both high- and low-voltage elements of the single action potential wave is often exceedingly regular over a period of several hours. We have the distinct impression that we are then studying two processes, developed to their maxima, which do not undergo any such progressive changes with time as would indicate true fatigue.

SUMMARY

By a direct method of analysis the low-voltage elements of the action-potential wave in nerve have been studied, and the voltage and duration of the negative after-potential have been approximately determined. The main observations, in part confirmatory of previous conclusions, are as follows:

1. In resting nerve the low-voltage elements are very much reduced or absent; after activation of the tissue by a period of repetitive stimu-

lation they become highly developed so that the electrical discharge from the negative after-potential may be from 4 to 10 times as great as that from the initial high-voltage "spike."

2. The negative after-potential, as well as the "after-positivity" are very much reduced at low temperatures (5°C. or below), even though the nerve is continually activated by repetitive stimulation.

3. During oxygen-lack the low-voltage elements, both positive and negative, disappear after one to two hours, so that the action potential wave consists of the high-voltage phase alone. The latter may continue for more than an hour after the suppression of the low-voltage phase. Upon the readmission of air the low-voltage elements soon reappear, and become very highly developed, often rising far above the values previously observed in the fresh nerve. The high-voltage phase shows no such behavior, merely returning to, or nearly to, its original value.

4. Direct analysis of the electrical discharge from two waves, separated by an interval (10σ) just longer than the relative refractory period, shows that while the high-voltage phases of the two waves come through without diminution, the low-voltage elements interfere over all, or nearly all, of their extent, so that the electrical discharge from the negative after-potentials of the two waves together is less than double that given by a single wave alone.

The differing behaviors of these two elements of the action potential wave are interpreted to mean that they are the electrical signs of two separate processes. The nature of the two processes which can show such relationships is discussed. It is concluded that the low-voltage after-potentials are the electrical sign of an active second process, restorative in nature, which builds the nerve back to its original state, possibly by replenishing the stock of some material whose breakdown is responsible for the high-voltage phase. This first process, underlying the high-voltage phase, is relatively anaerobic, and slightly affected by various physical and physiological changes. The second process is relatively aerobic, and is sensitively related to a variety of factors.

This conception is similar to the idea long ago proposed by Hering (1884) in explanation of the "after-positivity" after repetitive stimulation, but differs from that suggested recently by Levin (1927). The latter believes that the after-potentials arise from an accumulation of chemical metabolites during activity. To us the evidence suggests that the mere presence of such products has no effect upon the action potential wave, although they may affect the injury potential, but that the after-potentials only arise when these substances are being removed, or, more probably, when a new store of precursor substance is being formed, by direct chemical action.

A possible relationship between such chemical changes and the phys-

ical state of the membrane is suggested, according to which fluctuations in the pH of the tissue affect the dissociation state of an ampholyte near its isoelectric point, and so alter the value of the membrane potential by changing the membrane charge.

We are indebted to Mr. A. C. Downing for the constructions and adjustment of the galvanometer used in this study. We also wish to acknowledge the interest and aid of Mrs. Doris Preisler, who has assisted us in some of the experiments.

ADDENDUM. Through the courtesy of Dr. H. S. Gasser, who has very kindly placed his apparatus and personal services at our disposal, the general validity of the observations here reported on the effects of oxygen-lack has been confirmed by the oscillograph in his laboratory. In a number of experiments, both with bull frogs and green frogs, we have examined the effects of oxygen-lack upon the after-potentials of the α fibers of the A group, and have consistently observed a marked differential suppression of the after-potential, even in unpurified tank nitrogen, with a very prominent post-asphyxial augmentation.

This suppression is particularly striking in a second or third period of asphyxia, when the large after-potential, occurring after the preceding period in nitrogen, may almost completely disappear in twenty minutes. Until very late in asphyxia, however, the oscillograph shows some trace of a low voltage negative element enduring for 15 to 30σ. Its area is apparently too small to be detected by the method of analysis used in the present paper, whose experimental error, it should be stated, may be as much as 20 per cent toward the end of asphyxia.

The spike and after-potential areas have been approximately determined in several sets of oscillograph records. The data from a second asphyxia in one experiment came out as follows, areas being given in "millimeter-sigmas," calculated for four panel amplification.

	AREA OF SPIKE	AREA OF AFTER-POTENTIAL	DURATION OF AFTER-POTENTIAL
Before N_2	1635	1316	110σ
12 min. in N_2	1635	659	60
40 min. in N_2	1186	88	15

At 40 minutes in N_2, when the spike was still 75 per cent of the normal area, the after-potential, much reduced in duration, had fallen to 7 per cent of its original value, being now only 8 per cent of the total electrical discharge.

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STUDIES ON IMMUNITY TO RATTLESNAKE VENOM (CROTALIN)

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Before attacking the various phases of the problems of the physiologic mechanism of immunity a logical requirement would seem to be comparatively complete knowledge of the physiologic behavior of a given antigen. This requirement is seldom fulfilled. We can give no physiologic explanation for the cause of death in many diseases and infections. The reason for this is apparent when one considers the multiplicity of problems involved in such an investigation. If bacteria are concerned the pathogenicity of a given strain may vary greatly and with strains of equal virulence the susceptibility of the host is always an uncertain factor. This, with the varying length of time required for the manifestations of symptoms, increases the difficulty of obtaining decisive information on the physiologic processes involved. During our investigation of the physiologic properties of rattlesnake venom we were struck with its unique possibilities as an antigen in a study of the physiology of immunity.

The distinct advantage of this toxin in the study of immunity lies in the multiplicity of its striking actions, many of which can be measured. When injected intravenously into dogs and rabbits a profound fall in blood pressure follows which is accompanied by a marked increase in the volume of erythrocytes. Large doses result in multiple diffuse hemorrhages throughout the body and in a nonimmune or partially immune animal these manifestations are accompanied by a distinct group of symptoms. When rattlesnake venom is added to perfused organs there follows maximal contraction of the uterus of the virgin guinea pig and rapid loss of contractile power in the case of the rabbit heart. Any or all of these manifestations can be used as criteria in the study of the development of immunity (Essex and Markowitz, 1930).

In the present report we shall be concerned with methods of titrating the degree of immunity, the rate of development of immunity, the question of cellular immunity, and other problems in the investigation of which most of the manifestations mentioned are utilized as test objects.

Although in some respects crotalin may leave something to be desired as an antigen for use in the study of immunity yet there are sufficient points in its favor to outweigh the acknowledged disadvantages. All the facts accumulated on the physiologic aspects of immunity to one antigen may not be true of another; however, it is probable that the fundamental principles underlying the physiology of immunity are sufficiently similar to make the investigation of immunity to crotalin of distinct value.

I. A TECHNIC FOR ASSAYING THE ANTIHEMOLYTIC TITER OF RATTLESNAKE ANTIVENIN. There are many methods for titrating the antibody content of a sample of serum but whereas such investigations as the agglutinin titer and the bacteriolytic property of a serum may be useful in studying immunity to bacteria, they can obviously not be employed in the case of an antigen like rattlesnake venom. It has been shown in animals immunized to crotalin that there need be little correspondence between the precipitin titer of immune serum and its antibody content as determined by the L + dose on pigeons. This method is satisfactory but has several disadvantages. It is cumbersome, requiring a large number of pigeons. It is impossible to estimate the antibody content of serums that are not highly immune. Finally small fluctuations in the immune titer are detected with difficulty and only following the use of a very large number of pigeons. For the assay of antivenin for clinical injection the pigeon method should be satisfactory if enough birds are used.

In this paper we wish to report a technic for investigating the antivenin content of a serum. The method is convenient, rapid, and accurate. Possibly it can be applied to certain other venoms and toxins. Our method is based on the principle that the first phase of the hemolytic action of crotalin is to expand the erythrocytes so that when a specimen of heparinized blood is treated with traces of crotalin and centrifuged, the ratio of corpuscles to plasma will be found markedly increased. This increase in the volume of the sedimented erythrocytes is actually due to increased volume of the individual cells which, as can be observed microscopically, have lost their biconcave form and become spherical. In order to be certain that the result was not due to increased viscosity of the plasma with a resultant delay in the rate with which corpuscles are sedimented while centrifuged, with Baldes we have investigated the influence of crotalin on the viscosity of heparinized blood plasma. We found that crotalin had no appreciable effect on the viscosity of this medium.

Early in our research we found that blood from an immune animal did not react to crotalin by an increase in the volume of the erythrocytes (Essex and Markowitz, 1930). This led us to investigate the possibility of using this method for titrating the antivenin content of blood. It may be considered that in our method we are employing an incomplete hemolytic end-point which is arbitrarily assumed to have occurred when the least

dose of venom is found that will expand the erythrocytes 10 per cent under standard conditions. As will be shown later in this study, there is reason to believe that the antihemolytic titer of a serum, as measured in this manner, is an accurate index of the immunity of the organism to the lethal effects of the venom.

Before the expansion of the erythrocytes could be used as a measure of immunity it was necessary to establish a normal standard for comparison. By a series of experiments it was found that when 0.01 cc. of 0.01 per cent crotalin is added to 1 cc. of fresh, heparinized normal dog blood and allowed to stand at room temperature (25° to 26°C.) for fifteen minutes and then spun in a hematocrit tube for thirty minutes at 3,000 revolutions a minute, the volume of the corpuscles will be about 10 per cent greater than in a sample untreated with crotalin. Under these conditions there is practically no hemolysis. We have therefore adopted as our standard the smallest amount of venom necessary to increase the corpuscular volume just 10 per cent under the conditions mentioned. For determining this figure the routine procedure is as follows: 1, to each of eight small vials 1 cc. of fresh heparinized blood to be tested is added, care being taken to agitate the container before withdrawing the blood each time; this is obviously necessary if the suspension of erythrocytes is to be uniform; 2, the first and eighth tubes are kept as controls, but to the second to the seventh tubes, inclusive, graduated quantities of crotalin are added, beginning with doses which by previous experience are known to be too small to affect cell volume; 3, the crotalin is measured from a 0.1 cc. capillary buret graduated in 0.002 cc.; when introduced the crotalin is made to adhere to the walls of the tubes above the blood to be tested so that each sample of blood may later be mixed with the crotalin at the same instant; after the crotalin is added a cork is placed in each tube; 4, four tubes are taken in each hand and shaken gently; 5, the tubes are allowed to stand with occasional shaking for about eight minutes when a sample of blood from each tube is drawn into a hematocrit; it has been our experience that the best results are obtained if an elastic band is placed over the ends of the hematocrits before putting them into the hematocrit harness, and 6, exactly fifteen minutes after the crotalin was mixed with the blood the hematocrits are placed in the centrifuge and spun at 3,000 revolutions a minute for exactly thirty minutes; the percentile increases in the volume of the erythrocytes are charted on graph paper against the dose of venom, and the quantity of venom required to increase the volume just 10 per cent is obtained by interpolation.

With blood from normal dogs the figure was surprisingly constant as shown by repeated titrations which were good duplicates. The amount of venom required to produce the 10 per cent increase in the volume of erythrocytes varies with different dogs. The amount required for the blood

from fifteen normal dogs which we have studied ranged from 0.005 to 0.01 cc. of 0.01 per cent crotalin. When immune blood was employed the blood was titrated with stronger venom to avoid dilution, but the figure was always reduced to the same denominator, that is, 0.01 per cent crotalin. The venom employed was of such a strength that about 0.01 cc. was necessary to produce the desired result.

A reasonably immune dog requires 0.010 cc. of 1 per cent venom. In terms of 0.01 per cent venom this figure becomes 100 times as great. Therefore the titration figure for this dog becomes 1,000. That is, the blood of this animal requires 100 times the maximal normal dose to expand the corpuscles 10 per cent. It is not necessary to incubate the blood before it is drawn into the hematocrit tubes. During the spinning the centrifuge may develop changes in temperature which nullify such a precaution. The experiments were performed during the season when the temperature of the building was approximately 26°C.

In order to estimate the immune titer of rabbit blood we have adopted the method as follows: 0.9 cc. of blood from a normal dog is added to each of eight vials, to each of which is also added 0.1 cc. of the heparinized rabbit plasma. The mixture is then titrated in the usual manner. Control titrations in which we used normal rabbit plasma have given normal values.

In order to estimate the antibody content of a sample of blood from an immune dog that has recently been injected with a large dose of venom, it is unwise to titrate the whole blood in the usual manner, for the reason that the erythrocytes may not have recovered from any possible changes due to the venom. Accordingly, plasma is obtained, whose immune titer is estimated as in the case of rabbits. We have made it a rule not to titrate the blood of a given dog until at least three days have elapsed following the last injection of venom. If such information is necessary before this time the plasma is titrated by adding it to normal dog blood. The whole blood from an etherized animal should not be used.

With these simple precautions the method has been found very sensitive. We have obtained the same titer on a given sample of immune blood or plasma time and again, the extreme variations in the figures being less than 10 per cent.

As will be pointed out subsequently, the erythrocytes of an immune specimen of blood are as susceptible to the hemolytic action of the venom as are those from a normal dog. It is therefore an accurate procedure to estimate the immune titer of a specimen of blood by spinning it, and adding 0.1 cc. of its plasma to 0.9 cc. of blood from a normal dog.

In all these experiments we have prepared our dilutions of crotalin for titration from a stock sample of 5 per cent rattlesnake venom suitably preserved in glycerin and kept in the refrigerator. The dog that supplied

us with specimens of normal blood was one that was reserved for this purpose.

Under the conditions of our experiments the behavior of the erythrocytes is reasonably constant. When an insufficient quantity of venom has been added to affect the corpuscles the corpuscular reading is a little lower than the controls, this being an effect of dilution. A larger dose of venom will increase the corpuscular reading about 10 per cent if the concentration of venom is the proper one. There should be little, if any, hemolysis in this tube. The next dose of venom will expand the erythrocytes 15 to 20 per cent. There will be hemolysis in this tube. Subsequent increases in the quantity of venom do not increase but decrease the ratio of corpuscles to plasma. The extent of the hemolysis partially explains this result. In addition the result is due to the fact that the corpuscles probably shrink just before they hemolize. The results are graphed, and the amount of venom required to cause the 10 per cent increase is obtained by interpolation.

In our experiments the titration figure of a sample of blood is referred to as the 10 per cent dose, or the antihemolytic titer. By this is meant the least quantity of venom that will expand the erythrocytes of 1 cc. of dog blood 10 per cent under the standard conditions employed by us. The figure is always expressed in terms of 0.01 per cent rattlesnake venom. A word is necessary regarding the length of time required to centrifuge the hematocrit tubes before constant readings are obtained. In the case of normal blood spinning for half an hour at 3,000 revolutions a minute has, in our experience, given values which cannot be reduced by respinning. When blood is treated with crotalin, however, constant values cannot be obtained except in the control tubes, since although the corpuscles have been sedimented they are still under the influence of venom and are changing their volume constantly.

The outstanding advantages of this method for titrating the antihemolytic power of blood lies in its rapidity, its simplicity, and its accuracy. When once the experimenter has become familiar with the technic it is a simple matter to accumulate many observations rapidly.

II. Redevelopment of immunity to crotalin in previously immunized animals. By the use of the technic described, we have made observations on the rate of development of antibodies in dogs that have been previously immunized to crotalin and dogs that have not. The results of this study may be summarized by stating that when dogs have become immune following a number of intravenous injections of crotalin, they promptly begin to lose the immunity if these injections are discontinued. In about ninety days the antihemolytic titer is little, if any, higher than normal. If the dogs are again treated with a series of injections of crotalin, immunity is developed much faster than when the same

injections are given to control dogs, and the degree of immunity is much greater.

In the series here reported, the animals were injected with 0.01 cc. of 2 per cent rattlesnake venom for each kilogram of body weight three times a week. If the antihemolytic titer was to be investigated the blood was taken three days after the last injection. Whereas control dogs showed practically no immunity in three weeks, the previously immunized animals showed very high values.

The greatest immunity we have ever observed in dogs occurred when a previously immunized dog had been given nine such injections of crotalin.

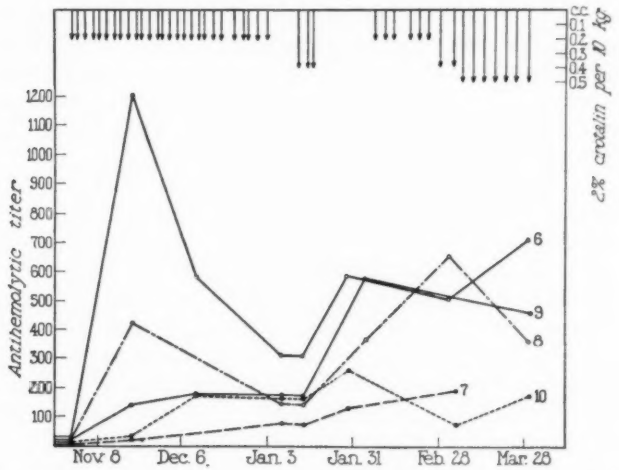

Fig. 1. A graphic representation of the immunity developed in previously immunized dogs (*6, 8, 9*) as compared to that developed in dogs not previously immunized (*7, 10*). The arrows indicate the number of injections, their length indicates the dose given, and their position the approximate dates of injections.

Control dogs following a parallel course of injections showed about one-fiftieth of the immunity (fig. 1).

When the same dose of venom was injected repeatedly the immune animals soon began to lose some of their immunity whereas the normal animals gained steadily. Increasing the amount of crotalin injected again revealed a similar difference in the reaction manifested by the previously immunized dogs.

We have no explanation for the tremendously more rapid increase in immune bodies in previously immunized animals. One possibility is that there is storage of antibody in some organ. A more likely explanation is that a mechanism for coping with the particular antigen has already

developed in the organism, whereas in normal animals it takes a considerable time for elaboration of the mechanism.

The observation just recorded is in line with that of Dean and Webb, who stressed its great significance. They studied the development of precipitin in the blood of rabbits that were being injected with horse serum. Following the injection of horse serum, antibodies could not be detected in a normal rabbit until the tenth day. In previously immunized animals a sharp rise in the precipitin titer began to occur on about the third day after the injection; it occupied three to five days and remained at a high level for four to six days.

Wassermann, according to Zinsser (1927), working with typhoid bacilli made a comparable observation. A series of rabbits was immunized to this organism until a high agglutinin titer was produced. They were kept until the titer had returned to normal and no agglutinins could be found. He re-inoculated them with typhoid bacilli along with a series of controls. In the previously immunized series a high titer rapidly developed whereas that of the controls remained at a much lower level.

Commercial houses engaged in the manufacture of diphtheria antitoxin are familiar with this principle in a practical way. They know that the best producers of antitoxin are animals who at some stage previously in their life have acquired immunity to diphtheria toxin.

Although we have been unable to demonstrate an actual cellular immunity to rattlesnake venom in the blood-free tissues of an animal, nevertheless some change occurs in the tissues of an animal during immunization to a given antigen whereby the animal can subsequently produce antibody at a much more rapid rate than normal when the need for this antibody arises.

Summary. The rate of development of immunity and the extent of immunity that occurs following a number of injections of crotalin are much greater when the injections are made into animals that have previously been immunized but have lost their demonstrable immunity. This observation amply confirms similar observations made when horse serum and typhoid bacilli were used as antigens.

III. Sensitivity of erythrocytes of immune dog. After it had been demonstrated that comparatively large doses of crotalin are required to produce expansion of the erythrocytes from an immune dog, the question was raised as to whether the erythrocytes themselves were resistant to the action of the venom, or whether they were protected solely by the antibodies in the plasma. The following experiment, which has repeatedly given the same result, has demonstrated that the erythrocytes of an immune dog are in every respect as vulnerable as those of a normal dog.

To obtain an answer to the question just raised, a logical procedure would be to interchange the plasma and corpuscles of a normal and im-

mune dog. After the addition of the same amount of crotalin to each tube and centrifugalization it would be evident if the erythrocytes of the immune dog were immune, in which event they would not increase in volume. In this experiment the following procedure was adopted. A sample of blood was drawn into heparin from each dog. The tubes were centrifuged for twenty minutes and the volume of erythrocytes in both tubes was about 7.5 cc. Another tube was removed from each dog and treated in the same manner except that 0.01 cc. of 1 per cent crotalin was added to each tube. After centrifuging, the erythrocyte volume of the normal sample was 11.5 cc. whereas that of the immune sample was the same as normal. A third sample was taken from each dog; the plasma was removed and the corpuscles washed twice in physiologic sodium chloride solution. About 2 cc. of the erythrocytes were lost from each tube in the washing process. The plasma from the normal dog was added to the erythrocytes

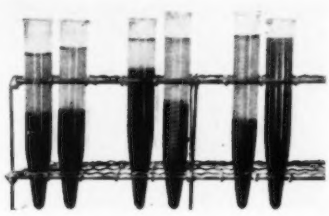

Fig. 2. The first two tubes represent centrifuged specimens of blood from normal and immune dogs respectively. The middle pair of tubes illustrate the effect of adding crotalin to blood from these dogs. The last pair of tubes show what happens when crotalin is added following the transposing of the plasma.

of the immune dog and the plasma from the immune dog was added to the erythrocytes of the normal dog, after which 0.01 cc. of 1 per cent crotalin was added to each tube. After centrifuging twenty minutes the volume of erythrocytes in the tube containing the immune plasma was 5.5 cc. and the volume of the erythrocytes in the tube containing the normal plasma and the erythrocytes of the immune dog was 8 cc. (fig. 2).

Another series of experiments was performed on blood from an immune dog the titer of which had been previously determined by the antihemolytic method, that is, the amount of crotalin required to produce an increase of 10 per cent in the volume of the erythrocytes. The titer of this dog was 700. After the corpuscles had been separated from the plasma they were washed twice in normal plasma and then suspended in plasma from a normal dog. Repeated titrations of the mixture gave normal titers.

From these experiments it may be concluded that the erythrocytes do not possess immunity to crotalin, that the immunity resides exclusively in the plasma.

IV. Comparison of immunity as indicated by antihemolytic titer and blood pressure. In part I of this paper we have described a method for assaying the humoral immunity to crotalin which develops in rabbits and dogs, and we have reported on the immune titers of a series of animals so measured. Although this method is reliable in determining the degree of humoral immunity and can be used with confidence in estimating the antibody content of immune serum, yet the question as to whether the titers thus obtained represent a comparable immunity to other actions of the venom, requires further investigation. In order to shed light on this problem the experiments reported here were undertaken.

In a previous paper (Essex and Markowitz, 1930) we have pointed out that the blood pressure of an immune dog is practically unaffected by an intravenous injection of a lethal dose of venom for a normal dog. An effort was not made at that time to determine the degree of immunity quantitatively. This was necessary in the present investigation since we wished to compare the degree of immunity by the response of the blood pressure with that indicated by the antihemolytic titer. The results of this comparison, although not strictly comparable, are sufficiently so to warrant accepting the antihemolytic titer as a fair indication of the animal's immunity.

Methods. Before undertaking such a study on immune dogs it was desirable to establish a normal standard for the action of crotalin on the blood pressure. The results of a series of experiments on nonimmune, etherized dogs have shown that 0.01 cc. of 2 per cent crotalin for each kilogram of body weight injected intravenously for a period of one minute produces a marked fall in blood pressure with complete recovery in ten to forty-five minutes (fig. 3). Experiments on normal dogs have likewise shown that 0.06 cc. of 2 per cent crotalin for each kilogram is usually a fatal dose.

A series of immune dogs was prepared for this study by graduated intravenous doses of crotalin. Each animal was given an initial injection of 0.01 cc. of 2 per cent crotalin for each kilogram. This was repeated three times a week for six weeks when it was increased to 0.04 cc. The first two injections of this magnitude were tolerated without symptoms, but the third produced the typical reactions following crotalin poisoning. It was then necessary to reduce the dose to twice the initial injection for two weeks. Subsequently the injections were gradually increased until all of the animals were receiving, without symptoms, from five to six times the initial dose at the end of four months. At this time the antihemolytic titer was obtained for each individual of the series after which the effect on the blood pressure was determined.

Results. When a dog has been immunized according to the program just outlined an injection of 0.01 cc. of 2 per cent crotalin for each kilogram under standard conditions of anesthesia and rate of injection, does not

produce a fall in blood pressure. It requires many times this amount to cause even a temporary depressor response. In these experiments we have as a routine injected six times the initial immunizing dose, or 0.06 cc. of 2 per cent crotalin for each kilogram over a period of one minute. The effect on the blood pressure requires several minutes for complete recovery. The dogs that showed the least immunity as measured by blood pressure showed the lowest degree of immunity as judged by the antihemolytic titer. The converse of this statement is also true since the blood pressure of dogs which showed the greatest degree of immunity by the antihemolytic test was practically unaffected.

The data on this series of experiments are briefly summarized in table 1.

TABLE 1

Immunity as measured by antihemolytic titer and blood pressure

DOG	WEIGHT	2 PER CENT CROTALIN INJECTED	ANTIHEMOLYTIC TITER*	EFFECT ON BLOOD PRESSURE	COMMENT
	kgm.	*cc.*			
1	20.5	0.2	6	Profound fall	Recovery in forty-five minutes; normal dog
2	15	0.9	53	Decided fall	Recovery in seven minutes
3	8	0.48	110	Slight fall	Almost immediate recovery
4	17.5	0.05	255	Slight fall	Almost immediate recovery
5	22.5	1.4	365	Slight fall	Almost immediate recovery
6	23.5	1.41	580	Very slight	Immediate recovery
7	12	1.4	700	Very slight	Immediate recovery

* The titration figure referred to in each case indicates the degree of humoral immunity as measured by the amount of crotalin required to produce a standard increase in the volume of the erythrocytes.

The effect of 0.01 cc. of 2 per cent crotalin for each kilogram on the blood pressure of a nonimmune dog is so invariable and characteristic that little doubt remains that an animal whose blood pressure is only temporarily depressed by many times such an intravenous dose possesses considerable immunity. The blood pressure tracing of dog 1 which was nonimmune shows a decided fall with complete recovery long delayed. When the blood pressure of dog 1 is compared with that of dog 2 which was given an injection six times as great for each kilogram it is quite evident that dog 2 possessed a fair degree of immunity. After an injection of what is usually a lethal dose the blood pressure of this dog fell precipitately to about 50 per cent of the normal, but complete recovery had occurred in about seven minutes. As determined by the antihemolytic method the immune titer of this dog was 53 which was the lowest of the series (figs. 3 and 4). Likewise when the effect of the standard dose of

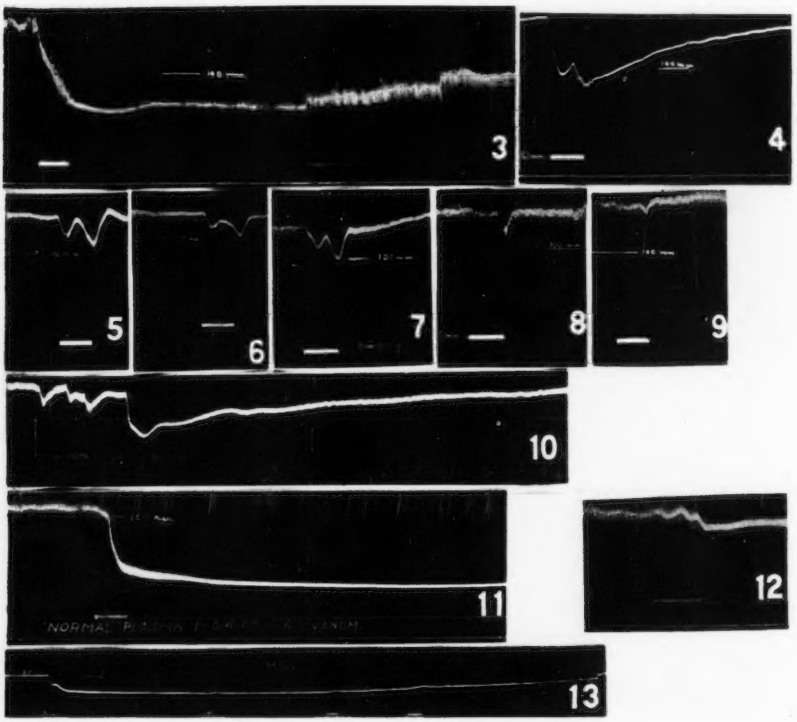

Fig. 3. Dog 1. Weight 20.5 kgm. The response of the blood pressure of a normal dog to an intravenous injection of 0.01 cc. of 2 per cent crotalin for each kilogram. In this case recovery of the blood pressure to the original level required forty-five minutes. Antihemolytic titer, 6.

Fig. 4. Dog 2. Weight 15 kgm. The effect of 0.06 cc. of 2 per cent crotalin for each kilogram given intravenously to a dog that showed a low order of immunity. Antihemolytic titer, 53.

Fig. 5. Dog 3. Weight 8 kgm. The response of the blood pressure of an immunized dog to an intravenous injection of 0.06 cc. for each kilogram. According to the blood pressure this animal possessed more immunity than dog 2 which is shown also by the antihemolytic titer which was 110.

Fig. 6. Dog 4. Weight 17.5 kgm. When 1.05 cc. of 2 per cent crotalin had been given intravenously to this dog, only a slight fall resulted with complete recovery almost immediately. Antihemolytic titer 255.

Fig. 7. Dog 5. Weight 22.5 kgm. The result following the injection of 1.4 cc. of 2 per cent crotalin into this dog was quite comparable to that for dogs 3 and 4. Antihemolytic titer 365.

Fig. 8. Dog 6. Weight 23.5 kgm. An injection of 1.41 cc. of 2 per cent crotalin for each kilogram had little effect on the blood pressure of this dog. Antihemolytic titer 580.

venom on the blood pressure of dogs 3, 4, and 5 is compared with that of the preceding dogs it is apparent that the former possessed a higher degree of immunity since there is only a temporary effect. As judged by the response of the blood pressure the immunity of these three dogs appears to be quite comparable but the antihemolytic titers were respectively 110, 255, and 365 (figs. 5, 6, and 7). However, if we continue with comparisons the blood pressure of the dogs showing the highest titers likewise were the most resistant. Thus dog 6 had a titer of 580 and the effect on the blood pressure of the standard dose was nil (fig. 8). Almost twice the standard dose or almost two lethal doses produced practically no depressor response in dog 7 and the antihemolytic titer of this dog was 700 (fig. 9).

Comment. A superficial inspection of the results of the experiments just described is sufficient to indicate a relative correspondence between the behavior of the blood pressure and the antihemolytic titer. This correspondence is most striking in the case of the least immune and the most immune dogs. The correspondence in the case of the dogs that stand in the intermediate position is not so close. When one considers the complicated nature of the mechanism of the blood pressure it is not surprising that absolute correspondence with the antihemolytic titer was not obtained in all cases. The limitations of the blood pressure as an accurate guide to the degree of immunity are quite obvious. However, it is capable of revealing whether much or little immunity exists but it appears to be incapable of showing the finer gradations of immunity. When the results of these experiments are considered from all points of view we feel that it is safe to conclude that the antihemolytic titer is a fair indication of the immunity possessed by the animals studied.

Blood pressure and passive immunity. During these experiments the opportunity presented itself of testing the prophylactic and therapeutic efficacy of highly potent immune serum against the injection of a large

Fig. 9. Dog 7. Weight 12 kgm. The result of an intravenous injection of 0.11 + cc. of 2 per cent crotalin for each kilogram (nearly twice a lethal dose) into a highly immune dog. Antihemolytic titer 700.

Fig. 10. Dog 8. Weight 4.5 kgm. After the intravenous injection of 15 cc. of immune rabbit plasma the animal received 0.4 cc. of 2 per cent crotalin with the usual profound fall resulting, but recovery was rapid and complete. Antihemolytic titer of the rabbit plasma 7,000.

Fig. 11. Dog 9. Weight 6.3 kgm. Control for experiment in figure 10 which shows the effect of 15 cc. of normal rabbit plasma plus 0.4 cc. of 0.2 per cent crotalin.

Fig. 12. Dog 10. Weight 13 kgm. Fifteen cubic centimeters of the same immune rabbit plasma were incubated at room temperature with 0.4 cc. of 2 per cent crotalin for fifty minutes. At the end of this time the mixture had a large quantity of flocculent precipitate, and on injection, the characteristic immediate effect is not evident.

Fig. 13. Dog 11. Weight 3.7 kgm. After an injection of 0.13 cc. of 4 per cent venom with usual effect a total of 14 cc. of commercial antivenin restored the blood pressure to normal.

dose of crotalin. A small dog weighing 3.4 kgm. was given 15 cc. of immune rabbit plasma intravenously. This plasma had a titer of 7,000. The injection of 0.4 cc. of 2 per cent venom caused a fall in blood pressure to 45 mm. of mercury within one minute, but sharp recovery immediately occurred; within five minutes the blood pressure was nearly normal again (fig. 10). This result is in striking contrast with the fatal effect of such a dose given to a control animal that had first been injected with 15 cc. of normal rabbit plasma (fig. 11).

In another experiment the immune rabbit plasma and the venom were incubated at room temperature for fifty minutes before injection into a normal dog. The characteristic immediate effect on the blood pressure was thus almost completely abolished (fig. 12).

In order to investigate the therapeutic effect of antibodies for crotalin a dog weighing 3.7 kgm. was given a fatal dose of crotalin intravenously. When the blood pressure had fallen to 50 mm., the animal was given two injections each of 4 cc. of reliable commercial preparation of antivenin. Each injection raised the blood pressure slightly. Six cubic centimeters of antivenin were again injected. In fifteen minutes the blood pressure had reached a level which was higher than before the venom was injected (fig. 13).

The experiments demonstrated that the union of crotalin with its antibody is not instantaneous, so that the injection of antivenin into a crotalinized animal does not instantly restore the blood pressure to normal. The rapidity with which this is accomplished undoubtedly varies with the dose of antibody administered. Conversely, when venom is rapidly injected into an animal that has been actively or passively immunized, there is a fall in blood pressure, the time required for recovery depending on the quantities injected and the degree of immunity possessed by the animal.

Evidence has not been produced in our experiments that the administration of a strong solution of antibody reverses the injury that has been brought about by the crotalin. Erythrocytes that have increased in volume under the influence of crotalin show no shrinkage following treatment with a large excess of antivenin. It would appear that the recovery of blood pressure which follows the injection of antivenin into a crotalinized animal is due to the natural compensatory mechanisms that can manifest themselves when further injury by the venom has been prevented.

Summary. Repeated injections were given to a series of dogs under standard conditions with graduated doses of rattlesnake venom and the immunity thus developed was measured by the reaction of the animal's blood pressure to a given dose of venom, and by the behavior of the erythrocytes in vitro. In general it was found that the antibody content of a sample of blood was roughly proportional to the immunity indicated by the response of the blood pressure.

The ability of immune serum and commercial antivenin to bestow passive immunity was demonstrated.

V. THE EFFECT OF CROTALIN ON THE PERFUSED HEARTS OF IMMUNIZED RABBITS. During our investigations the question arose as to the part played by the tissues of an immunized animal in protecting the animal from the lethal effects of an injection of venom. Clinically it is known that a patient may be immune to some bacterial infections although there may be no demonstrable antibodies in the blood for the responsible organism. This may be true for many diseases. It has been the custom to

TABLE 2

Rabbit 5 (weight 2.4 kgm.)

DATE	INJECTIONS	2 PER CENT CROTALIN INJECTED	COMMENT
		cc.	
December 14 to January 16	9	0.1	Decided reaction followed first four injections
January 18 to February 25	9	0.2	No reaction
March 1	1	0.3	No reaction
March 5 to 7	2	0.4	No reaction
March 8 to 11	2	0.5	No reaction
March 13	1	0.6	No reaction
March 15	1	0.7	No reaction
March 18	1	0.9	No reaction
March 22 to 25	2	1.0	No reaction
May 5 to 14	3	0.4	Considerable reaction followed the first two injections; much less after third
May 15	1	0.5	No serious reaction at this time or subsequently
May 20	1	0.5	
May 24	1	0.6	
May 26 to 27	2	0.6	
June 2 to 3	2	0.8	

explain this immunity either on the basis of Besredka's theory of immunity at the portal of entry or on the ability of cells that are free of blood and lymph to resist the effects of a poisonous antigen. The striking effect of crotalin on the perfused heart of the rabbit makes this organ particularly suited to such an investigation.

Although the results that we are reporting for rattlesnake venom need not apply to bacterial infections they are so definite as to be interesting from the point of view of the physiology of immunity. Since the heart may be considered as a portion of the muscular system the results in this paper may reasonably apply to the muscular system as a whole.

Methods. A series of rabbits of approximately the same weight was immunized to repeated intravenous injections of crotalin. Table 2 is a typical protocol of this series.

June 5 the animal whose experiments are recorded in table 2 was etherized and exsanguinated. (The blood was heparinized and the plasma was preserved for investigation of antibody content.) The heart was then perfused in the usual manner, the apparatus being arranged so that graphic records could be taken.

It was essential in these experiments to wash the hearts thoroughly to remove all traces of immune blood and lymph for which purpose, besides the initial washing to free them from gross blood, they were given a preliminary perfusion with 500 cc. of oxygenated Locke's solution. When this quantity had been perfused through the coronary vessels (which required about fifteen minutes) the perfusion fluid was changed to 1 liter of Locke's solution containing venom.

The antibody content of the blood of these rabbits was determined by the methods described in part I of this report which, in brief, is estimated on the basis of the least amount of venom that will cause a 10 per cent increase in the volume of the erythrocytes of a normal dog when 0.1 cc. of plasma from an immune rabbit is added to 0.9 cc. of the dog's blood.

As we have noted, the immunity of these rabbits was further tested by the ability of their plasma to protect dogs against the depressor action of intravenous injections of crotalin.

Results. Since we were chiefly concerned with the reaction to crotalin of hearts from immune rabbits as compared with hearts from nonimmune rabbits, the degree of immunity possessed by the former was of paramount consideration. When 0.1 cc. of the immune rabbit plasma was mixed with 0.9 cc. of blood from a normal dog it required from 100 to 400 times the concentration of crotalin required for normal blood to produce a 10 per cent increase in the volume of the erythrocytes. It has been calculated that 1 cc. of such immune plasma is capable of neutralizing at room temperature in thirty minutes 0.75 to 4.0 mgm. of undried venom. In the case of the rabbit whose injections are described the antihemolytic titer of its plasma was such that it required 0.007 cc. of 1 per cent venom to expand the erythrocytes of a normal dog 10 per cent of their normal volume when 0.1 cc. of this plasma was added to 0.9 cc. of dog's blood. Roughly speaking 1 cc. of this plasma can neutralize in thirty minutes at room temperature 0.6 mgm. of undried venom. The plasma of one of the rabbits of this series was as effective as the same amount of commercial antivenin in restoring the blood pressure of dogs following intravenous injections of crotalin. When given previous to the injection of venom it was equally effective in preventing the depressor action of injections of crotalin. As additional evidence that these rabbits possessed considerable immunity it might be

added that when 5 cc. of the plasma was mixed with 0.05 cc. of 5 per cent venom there occurred an obvious turbidity within one minute, and gross flocculation within thirty minutes. From this evidence it may be considered that the rabbits in question possessed a sufficient degree of immunity to make the results obtained of significance.

In our experiments with crotalin we have been unable to obtain a degree of immunity in dogs or rabbits comparable to that reported for diphtheria and tetanus toxins, the proper injections of which may protect an animal against thousands of minimal lethal doses. This may be due to the fact that crotalin produces its characteristic action immediately after injection whereas the toxins referred to require a latent period. So far we have been unable to induce immunity to more than 6 to 10 minimal lethal doses of crotalin.

At this point it should be recalled that when 0.1 cc. of 2 per cent solution of rattlesnake venom is added to 1 liter of the perfusion fluid (Locke's solution) the heart is affected in a reasonably constant manner; it becomes progressively enfeebled and edematous, and little blisters appear under the visceral pericardium, these being transudates of Locke's solution. In sixty plus or minus fifteen minutes the pulsations cease. Since it is not possible to determine the exact moment when pulsations cease on account of terminal heart block we have considered the end-point as the moment the pulsations are regular and barely lift the heart lever. At the same time we have kept records of the survival periods of the hearts subsequent to this point. This period was not altered by adding a small quantity (2.8 cc.) of normal rabbit plasma to the perfusing fluid. It is hardly necessary to add that when the heart of a rabbit is perfused without venom in the perfusing fluid it will survive in good condition for eight to ten hours.

Much to our surprise the hearts from immune rabbits perfused under the same conditions behaved identically. There was not the slightest indication that any one of the hearts from the immune rabbits was able to withstand the destructive action of 0.1 cc. of 2 per cent crotalin more effectively than the normal heart (fig. 14).

The question was raised at this time as to the degree of immunity a heart should possess to insure protection against the quantity of venom added in each case to the perfusing fluid. To decide this point experiments were performed in which normal hearts were perfused with venom according to the standard technic with the difference, however, that varying quantities of plasma from immune rabbits were added to the perfusing fluid previous to the addition of the venom. The experiment definitely indicated the great sensitivity of this method for detecting immunity. The heart of a rabbit weighs about 6 grams. The addition of much less than this quantity of immune rabbit plasma completely protected the hearts from the venom. Thus, when 2.8 cc. of immune plasma were added

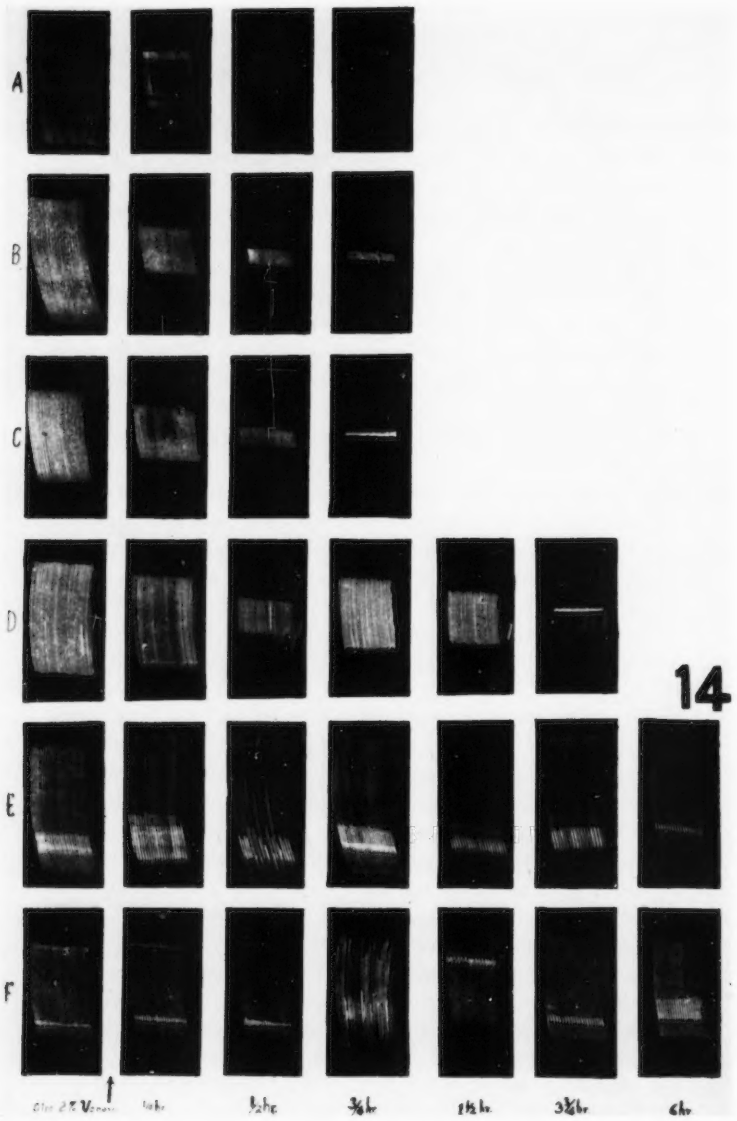

Fig. 14. Tracings of the perfused hearts of rabbits under the influence of a standard quantity (0.1 cc.) of 2 per cent rattlesnake venom in 1 liter of Locke's solution. The first column represents cardiac contraction prior to the addition of venom. *A* shows the rapid extinction of the heart beat of a normal rabbit heart following the venom. *B* illustrates the same effect when the heart is perfused in the case of a rabbit that has been immunized to crotalin. *C* shows the same effect in spite of the addition of 0.1 cc. immune plasma. *D* shows the protective effect of 0.35 cc. of the same immune plasma. Pulsations persist for three and three-quarters hours. *E* and *F* show the complete protection accorded by 1.4 and 2.8 cc. immune plasma respectively. Shortly after the addition of the venom the hearts showed temporary irregularity, but were beating vigorously at the end of six hours.

to 1 liter of Locke's solution the heart was completely protected, pulsating vigorously at the end of six hours when the experiment was terminated. During the experiment the pulsations manifested some diminution in extent. This change was temporary, the heart recuperating probably as a consequence of the gradual combination of the venom with the antibody. Similar results followed when 1.4 cc. of immune plasma was employed. With 0.35 cc. of immune plasma the heart pulsated for more than three and three-quarters hours. It was only when the quantity of immune plasma was reduced to 0.1 cc. that the protective power was inappreciable. The immune plasma employed was of about average potency as determined by our method. When 0.1 cc. of this plasma was mixed with 0.9 cc. of normal dog blood it required 0.009 cc. of 1 per cent venom to expand the erythrocytes 10 per cent. Roughly speaking 1 cc. of such a plasma will neutralize 0.9 mgm. of undried venom in half an hour at room temperature.

Comment. Seven experiments were performed in which the hearts of rabbits were perfused with Locke's solution containing the standard amount of venom. These hearts succumbed no more slowly than the hearts of normal rabbits. By adding varying quantities of the plasma from these immune rabbits to the perfusing fluid it was established that the degree of immunity possessed by the hearts of the immunized rabbits free of blood could not be greater than that possessed by 0.1 cc. of their plasma. In other words a 6 gram heart from an immune rabbit had no greater immunity than 0.1 cc. of its plasma. As a matter of fact, if the heart did possess the degree of immunity contained in 0.1 cc. of plasma, the protection against crotalin would be many times greater than the addition of this quantity of plasma to 1 liter of Locke's solution since the antibody in the cardiac muscle cells would be much more concentrated. The concentration may be obtained approximately by dividing the weight of the Locke's solution used (1000 gm.) by the weight of the heart (6 gm.) which would give a concentration in the heart of nearly 170 times that in the Locke's solution.

From these considerations it is fair to infer that the cardiac tissue of an immunized rabbit does not contain antibody for crotalin. In view of the fact that small quantities of immune plasma are capable of affording marked protection to the perfused heart it is perhaps fortunate for this research that the immunity developed was not as great as can be induced with diphtheria toxin. It is obviously impossible to wash out every trace of blood and lymph from a perfused heart and if the animals had been able to tolerate 1000 minimal lethal doses of crotalin, the merest trace of plasma in the heart would have been sufficient to lead to false conclusions.

That the degree of immunity produced in the rabbits was fairly extensive may be indicated by the observation that one of the animals possessed a plasma whose antibody content was as great as that of a reliable sample

of commercial antivenin for human injection. This product is concentrated from the original immune horse serum.

Summary and conclusions. Perfused hearts from rabbits possessing a relatively high degree of immunity to crotalin react as do normal hearts to the same dose of rattlesnake venom. It is evident that cellular immunity is wanting in the tissues of the heart and it is probable that the same is true of the musculature as a whole.

GENERAL SUMMARY

The venom of the rattlesnake is particularly suited as an antigen in the study of immunity because of the multiplicity of its measurable reactions. When injected into dogs or added to their blood in vitro this venom causes the erythrocytes to swell. Advantage has been taken of this fact in developing a new technic for assaying the antihemolytic titer of rattlesnake antivenin. The immunity of a given animal is measured in terms of the least quantity of crotalin necessary to cause a 10 per cent increase in the volume of the erythrocytes under standard conditions. This method is simple, rapid, and accurate and obviates the use of pigeons in assaying the antibody content of a given blood. By the use of this method a study has been made of the development of immunity to crotalin in dogs and rabbits. Dogs being immunized for the first time develop antibodies very slowly. When the injections of the venom are discontinued, their demonstrable immunity completely disappears in about ninety days. However, when injections are begun again antibodies develop in the dogs that were previously immune at a far more rapid rate and to a much higher degree than in control dogs being immunized for the first time. This is in agreement with similar results obtained by other workers who used horse serum and typhoid bacilli as antigens.

A study was made of the erythrocytes of immune dogs to determine whether they possessed resistance to crotalin or were protected entirely by the antibody in the plasma. It has been shown by transferring washed erythrocytes of an immune dog to plasma of a nonimmune dog, and vice versa, that the erythrocytes of an immune dog are as vulnerable as those of a nonimmune dog. These cells are protected solely by the antibody content of the plasma.

By a comparison of the immunity of the dog as measured by the antihemolytic titer and by the reaction of the blood pressure in response to a standard dose of crotalin it has been shown that a reasonably close correspondence exists. The blood pressure of immune dogs with high titers is practically unaffected whereas dogs with low titers show a very decided depressor response to the standard dose of venom. Passive immunity was conferred on dogs by injections of immune rabbit plasma and commercial antivenin. Both were effective in preventing the usual vasodepressor re-

action of the blood pressure of normal dogs following injections of crotalin. We have not found evidence that antivenin reverses the injury done by crotalin. It would appear that the recovery of the blood pressure which follows the injection of antivenin into a crotalinized animal is due to the natural compensatory mechanisms that can manifest themselves when further injury is prevented.

To determine whether cellular immunity to crotalin exists a study was made of perfused hearts of immune rabbits. Such hearts were incapacitated just as rapidly as hearts from nonimmune rabbits which indicates that cellular immunity is lacking in the tissues of the heart and probably the same is true of the musculature as a whole.

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ELECTRICAL MEASUREMENTS OF NEUROMUSCULAR STATES DURING MENTAL ACTIVITIES

VII. Imagination, Recollection and Abstract Thinking Involving the Speech Musculature

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Since Plato called thinking "an inner speech," nothing definite has been developed on the physiology of this relationship. Introspective accounts by Erdmann (1851), Geiger (1868), Stricker (1880) and others agreed that during mental activity there commonly occur feelings like those present in the musculature during actual speech, but left in doubt whether these feelings arise from peripheral contractions. This doubt persists to date. Dodge (1896) reports that after anesthetizing his tongue and lips with 20 per cent cocaine solution, "inner speech" is not disturbed, showing its independence from peripheral elements. Mechanical methods to record movements in the speech-organs during mental activities have brought divergent results in the hands of various investigators: Positive results, at least in a considerable proportion of tests, have been reported by Curtis (1899), Courton (1902), Tomor (1910), Wyczoikowski (1913), Reed (1916), Golla (1921), Clark (1922), but negative results by Krall (1925) and particularly by Thorsen (1915). The latter points out the inadequacy of previous methods used to record movements in the speech-organs and, employing Sommer's tridimensional analyzer, obtains magnifications up to 4.5. In spite of these improvements upon previously used methods, she finds that her experiments "throw no light on the actual mechanism of internal speech" and that they show "no qualitative correspondence between overt speech and implicit speech during the verbal formulation of the same ideas." Likewise after a comprehensive review of the whole subject, Schilling (1929) finds that investigations have not yet made it possible to say conclusively as to the bodily accompaniments of inner speech.

Accordingly it is certain that refined methods will be required to solve the present problem. We desire to enquire whether during certain mental activities there occur minute muscular contractions essentially similar in

pattern to those present during audible speech to the same effect. Two measures, not available until recent years, seem indispensable (Jacobson, 1930a, b, c, d; 1931a, b): 1, the method of extreme muscular relaxation, which is required to isolate the possible microscopic muscular contractions in the speech-organs during mental activities from other muscular contractions which commonly occur even in resting individuals if not trained to relax; 2, a sufficiently delicate recording apparatus, not affected by disturbances irrelevant to the problem.

METHODS. This second requirement, I have found, is not fulfilled even by the highly sensitive instrument previously described (Jacobson, 1930a). The irregular movements of the string shadow, present even when the input terminals of the amplifier are short-circuited or when the subject is in line but relaxed, are in some subjects comparable in voltage with the slight electrical changes in the speech muscles during mental activities. In order to remedy this failing, as well as to secure still higher magnification, another circuit is now employed (figs. 1, 2). The limitations of this circuit, in that amplification falls off for frequencies below about forty cycles per second, are shown in figure 2. I wish to thank the Bell Telephone Laboratories for their generosity toward the development of suitable equipment and, in particular, Messrs. H. A. Frederick and D. G. Blattner for various suggestions and aid.

Ideally, when the input of the amplifier is short-circuited, the string shadow should make a straight horizontal line on the photograph, indicating zero potential difference. The same should obtain with the subject in line during perfect relaxation. But during six months of continual experimentation, notwithstanding the use of specially designed filters, the effects of electrical currents adjacent to the laboratory proved discouraging. Most of the following measurements were made after the apparatus was removed to a location practically free from sources of electrical interference as well as of mechanical vibrations.

Months of detailed attention have been required in order to get the present apparatus to work satisfactorily. Numerous difficulties arise, as tested with the input on short-circuit; for example, from imperfect contacts, loose connections, undercharged batteries, "noisy" tubes, and poor ground-connections. When the difficulty is minor, records may be taken and the microvoltage of vibrations during periods when the input terminals are short-circuited or during control periods of relaxation is to be subtracted from the microvoltage of the vibrations occurring during test periods. In records taken with B. J. and A. T., the apparatus was in best condition and with the input on short circuit, the string was almost perfectly quiet.

Electrodes consist of fine platinum-iridium wire, pointed and inserted about one to two centimeters into the muscular tissues tested. In order

to prevent thermal electrical effects, the portion of this wire outside of the tissues is insulated with rubber tape, bakelite or glass.

A Sanborn string galvanometer is used in the following studies, excepting with B. L. The string tension as a rule is adjusted so as to record one centimeter deflection per millivolt p.d. applied to the string terminals.

During each period of tests, in order to calibrate the deflections of the string shadow when the amplifier is included in the circuit, a photograph is taken when an oscillating p.d. of one microvolt is applied to the input (fig. 3). In addition, during the later tests, a photograph is taken when the same calibrating microvoltage is applied in series with the electrodes in the relaxing subject.

The accuracy of the apparatus shown in figure 3 is readily checked. Using the amplifier previously described (Jacobson, 1930a, fig. 1), potential differences of 10 microvolts are applied in two manners: 1, with the oscillating current; 2, with the calibrating potentiometer already standardized (loc. cit., fig. 2; see also pp. 572, 573). Readings secured under the two sets of conditions show a substantial agreement. No attempt is here made to determine precisely the magnitude of error, but upon inspection, it appears to be under 5 per cent.

As heretofore, the click of a telegraph key serves as the signal to the subject to engage in a mental activity previously agreed upon. A second click is the signal to him to relax any muscular tensions present. Accordingly it is understood that the subject is to relax as fully as he can previously to the first and subsequently to the second signal.

So far as possible, the subject is not informed as to the purpose of the tests and of course no hint is given that he is in any way to use his speech-organs.

The instructions (see legend for table 1) are chosen to include not alone imagination and recollection, but the elements of other types of mental activity as well. For special control tests the instructions are "When the signal comes, do not bother to imagine" (control 7); or "Imagine Eiffel Tower" or "Imagine lifting a ten-pound weight with the right arm." The second of these instructions will probably arouse contractions in the ocular muscles (Jacobson, 1930c), while the last will probably arouse contractions in the right arm muscles, perhaps also in the ocular muscles (Jacobson, 1930a, b, d; 1931a, b). Accordingly, the latter two control instructions doubtless will excite imagination of some kind; but they will serve to test whether our previous conclusion that imagination involves contraction specifically within the muscle-groups which would be engaged during the actual performance, holds for the present instances. Obviously in such event, the results of the control tests should be negative.

Seven subjects are used in the present investigations, all, except one, more or less trained to relax. For subject B. L., one electrode is inserted

TABLE 1

Electrical measurements during imagination, recollection and thinking

Headings of the various columns will be understood from previous articles. Instructions are: A = Imagine counting. B = Imagine stating the date. C = Multiply 19 x 19. D = Multiply 23 x 22. E = Multiply 11 x 99. F = Recall "A rolling stone, etc." G = Recall "Star Spangled Banner." H = Recall any poem. I = Make up mind for tomorrow. J = Think of "Infinity." K = Meaning of "everlasting." L = Meaning of "incongruous." M = Think of "Ohm's Law." N = Think of "electrical resistance." O = Imagine speaking about holiday. P = Multiply 11 x 18. Q = Multiply 11 x 14. X = Do not imagine (control 7). Y = Imagine lifting weight with right hand. Z = Imagine Eiffel Tower.

Ideally, a column should appear showing the average value of V°_{2m} during imagination in each test; but such detailed measurement does not seem worth while, because of the great variation from subject to subject and from time to time. Instead, the last column contains figures indicating the microvoltage of a typical line V°_{2m} during imagination. No precise value attaches to the figures in the last column; they are intended only to convey some idea of the order of magnitude.

In many of the records (those of D. B., B. J., A. T., and some of B. E.) a condenser of 0.75 mf. inadvertently was left in shunt across the output of the amplifier. Correction of readings for the presence of this condenser would require a knowledge of the frequencies of the voltages impressed during imagination, but these frequencies are not determined with the type of electrodes used in present tests. The uncorrected readings are included in the table because their order of magnitude does not seem to differ essentially from values secured where no such corrections are needed.

INSTRUCTION	RESULTS OF TESTS			MICROVOLTS DURING TEST PERIOD		
	Number of tests	Positive	Negative	Minimum	Maximum	Representative
Subject B. L.						
A	5	5	0	3	233	6
Z	1	0	1	0	0	0
B	2	2	0	3	120	12
O	2	2	0	3	133	12
C	1	1	0	3	78	12
D	1	1	0	3	72	12
E	1	1	0	3	54	12
J	1	1	0	2	12	3
M	1	1	0			
N	1	1	0			
F	1	1	0	2	12	3
G	1	1	0			
H	1	1	0			
I	1	1	0			
X	5	0	5	0	0	0
Total tests A to O...	19	19	0			
Total controls.....	6	0	6			

INSTRUCTION	RESULTS OF TESTS			MICROVOLTS DURING TEST PERIOD		
	Number of tests	Positive	Negative	Minimum	Maximum	Representative
Subject B. E.						
A	1	1	0	4	42	20
G	3	3	0	2	100	24
O	1	1	0	4	90	24
P	1	1	0	4	60	30
Q	1	1	0	4	90	50
A	2	2	0	1	15	3
B	1	1	0	5	90	20
O	1	1	0	5	70	15
C	1	1	0	5	20	10
D	1	1	0	5	45	15
E	1	1	0	5	30	15
F	1	1	0	5	35	15
G	1	1	0	5	55	10
H	1	1	0	5	50	10
I	1	1	0	5	20	10
J	2	1	1	10	35	20
K	1	1	0	7	30	10
L	1	1	0	10	40	20
Y	4	0	4	0	0	0
X	3	0	3	0	0	0
Total tests A to O...	22	21	1			
Total controls.....	7	0	7			

INSTRUCTION	RESULTS OF TESTS			MICROVOLTS DURING TEST PERIOD		
	Number of tests	Positive	Negative	Minimum	Maximum	Representative
Subject A. T.						
A	1	1	0	1	12	6
B	1	1	0	2	21	6
C	1	1	0	2	36	9
D	1	1	0	2	12	6
E	1	1	0	2	8	5
F	1	1	0	1	2	2
G	1	1	0	2	3	3
H	1	1	0	1	4	2
I	1	1	0	2	3	2
J	1	1	0	0.5	2	2
K	1	1	0	2	2	2
L	1	0	1	0	0	0
M	1	1	0	4	7	5
N	1	1	0	2	2	2
X	11	0	11	0	0	0
Y	11	0	11	0	0	0
Total tests A to O...	14	13	1			
Total controls.....	22	0	22			

INSTRUCTION	RESULTS OF TESTS			MICROVOLTS DURING TEST PERIOD		
	Number of tests	Positive	Negative	Minimum	Maximum	Representative
Subject D. B.						
A	1	1	0	1	15	2
B	1	1	0	1	8	2
O	1	1	0	1	5	4
C	1	1	0	1	2	1
D	1	1	0	1	55	2
E	1	1	0	1	2	1
F	2	2	0	1	8	2
G	1	1	0	1	40	4
H	1	1	0	1	40	4
J	2	2	0	1	60+	5
I	1	1	0	1	5	2
K	1	1	0	1	10	2
X	4	1	3	0	0	0
Y	4	1	3	0	0	0
Total tests A to O...	14	14	0			
Total controls.....	8	2	6			

INSTRUCTION	RESULTS OF TESTS			MICROVOLTS DURING TEST PERIOD		
	Number of test	Positive	Negative	Minimum	Maximum	Representative
Subject B. J.						
A	1	1	0	0.5	9	1
B	1	1	0	5	30	10
O	2	1	1	2	28	8
C	1	0	1	0	0	0
D	1	1	0	3	28	8
E	1	1	0	2	30	10
F	1	1	0	1	30	3
G	1	1	0	3	4	3
H	1	1	0	1	4	2
I	1	1	0	3	30	10
J	1	1	0	1	30	5
K	1	1	0	2	30	10
L	1	1	0	2	30	3
M	1	1	0	1	7	2
N	1	1	0	0.5	23	5
X	10	0	10	0	0	0
Y	10	0	10	0	0	0
Total tests A to O...	16	14	2			
Total controls.....	20	0	20			

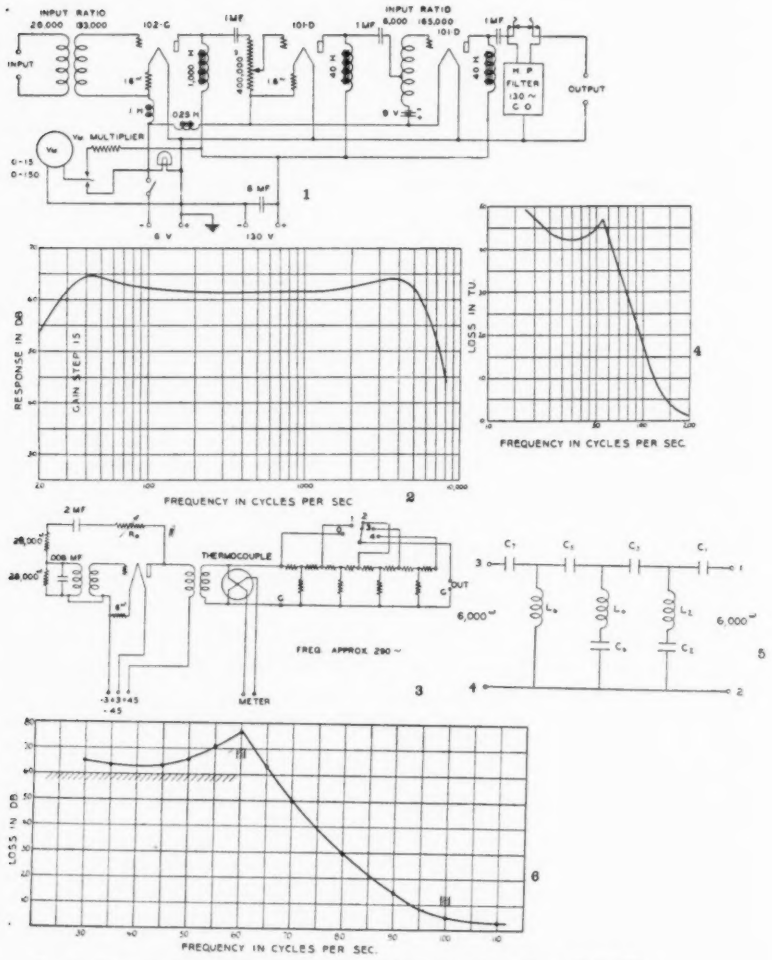


Fig. 1. *Circuit of amplifier no. 2.* Potentiometer has steps 0 to 22.

Fig. 2. *Curve showing response of amplifier no. 2* in DB as function of frequency in cycles per second (plotted logarithmically). The potentiometer is here set at step 15 and input impedance is 10,000 ohms while output impedance is 6,000 ohms. The definition of decibel (DB) or transmission unit (TU) as here used, where V_1 and V_2 are impressed across identical impedances, is, $N_{DB} = 20 \log |V_1/V_2|$. For example, 60 DB means that the voltage step-up is 1000.

Fig. 3. *Circuit containing oscillator used for standardizing.* When the rheostat R_0 is adjusted for a meter reading of 140 microamperes, setting the potentiometer at step 4 gives an output of 10^{-6} volts; at step 3, 10^{-5} volts.

in the tip of the tongue, while the other is inserted under the mucosa in the cheek on either side. To minimize electrical disturbances due to changing temperatures, the mouth is kept closed. Cotton is placed under the tongue to absorb saliva. But so long as salivation occurs, the string vibrates irregularly and the tests concerning mental activities have to be postponed. With sufficient practise, however, salivation diminishes or ceases during advanced relaxation, as shown by the cotton remaining almost dry for the hour of tests. Following an extraordinarily long period of training for this subject in relaxing the tongue and lips (Jacobson, 1929, chapter V), a straight base-line was secured in the physiological laboratory notwithstanding the electrical disturbances due to alternating and direct current in the building. Since it was necessary to minimize the effects of the alternating lighting circuit, two filters were inserted in the circuit between the amplifier output terminals and the galvanometer input terminals (figs. 4, 5, 6).

In the other subjects, electrodes inserted as above described for B. L. failed to yield a satisfactory base-line during attempted relaxation for reasons not fully determined. Therefore both electrodes are inserted in the mid-line of the under lip about one centimeter apart.

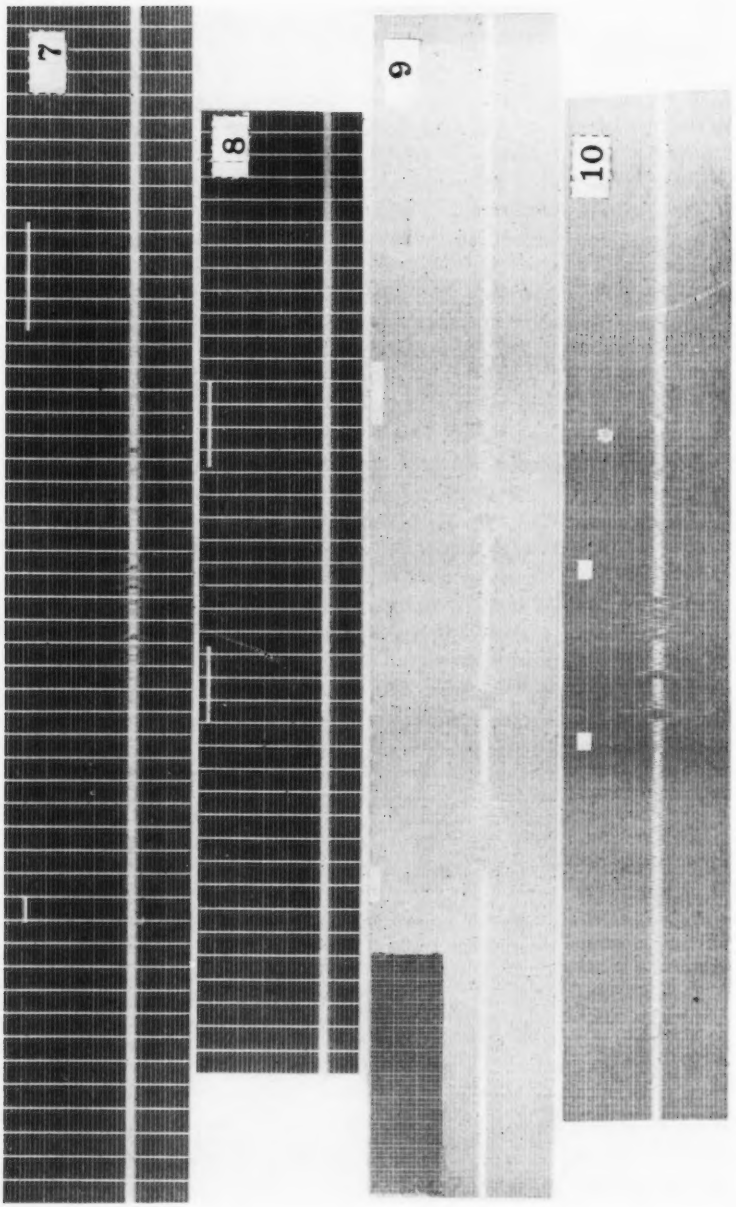
RESULTS. *When the electrodes are connected in the speech musculature of the trained subject, the string shadow is practically quiet during relaxation. But promptly after the signal is sounded to engage in mental activity involving words or numbers, marked vibrations appear, indicating action-potentials. Soon after the subject hears the signal to relax any muscular tensions present, the vibrations cease, and the string returns to rest* (figs. 7, 8).

Fig. 4. *Curve for filter "HP 130" in circuit shown in figure 1.* Shows energy loss expressed in transmission units (1 TU = 1 DB) as function of frequency in cycles per second (expressed logarithmically).

Fig. 5. *Circuit diagram of 60 cycle high pass filter,* designed to diminish interference from alternating current lighting circuit. When used, terminals 1 and 2 are joined to tube output terminals P and G of amplifier no. 2, while terminals 3 and 4 are joined to galvanometer terminals. The constants of this circuit are as follows:

RETARDATION COILS	INDUCTANCE (*h*)	CONDENSERS	CAPACITY (*mf*)
L_2	7.13	C_1	0.3962
L_4	7.13	C_2	0.9880
L_6	5.31	C_3	0.1981
		C_4	0.9880
		C_5	0.1692
		C_7	0.2950

Fig. 6. *Curve for filter shown in figure 5.* Shows energy loss in DB as function of frequency in cycles per second.


7
8
9
10

The series of vibrations during the mental activity occur in patterns evidently corresponding with those present during actual speech. For if the amplifier rheostat is set for a relatively low sensitivity and the instruction has been, "When the first signal comes, whisper as faintly as possible, 'One, two, three,' " three series of vertical lines are generally found on the photograph separated by a horizontal interval (fig. 9). If now the amplifier rheostat is changed to a sufficiently higher sensitivity and the instruction is this time to imagine or to recall counting but not actually to count, a quite similar photograph is secured (fig. 10). Likewise when verbal matters are imagined or recalled, such as a poem, records of action-potentials are secured resembling those from actual faint speech, but of considerably less voltage.

Fig. 7. *Photographic record of string deflections during thinking.* The subject, B. L., has been instructed, "Upon hearing the first signal, think of the meaning of the phrase, 'electrical resistance.' Upon hearing the second signal, relax any muscular tensions, if present." One electrode in tip of tongue; the other under mucosa in cheek. Interval between vertical time lines = $\frac{1}{5}$ sec. Distance between horizontal lines, 1 mm. = 3×10^{-6} volts.

This photograph shows between signal-marks groups of vertical lines, evidently due to action-potentials, which are absent in the periods of relaxation before the first signal and after the second signal. The microvoltage of these lines varies from about 1 to 30. We conclude that in the present test, muscle-fibers in the tongue of the subject contract as she thinks. (Her subjective report is in harmony with this conclusion.)

Fig. 8. *Photographic record of control test.* Conditions the same as in figure 7, excepting that here the instruction has been, "Upon hearing the first signal, do not bother to think." No evidence of action-potentials in the tongue here appears. Results are negative similarly with other subjects in tests where the instruction has been, "Imagine lifting a weight with the right arm" or "Imagine Eiffel Tower." Either of the latter two instructions are characteristically followed by action-potentials in muscles other than the tongue.

Fig. 9. *Photographic record of string deflections during counting.* The subject, B.E., has been instructed, "Upon hearing the first signal, whisper as faintly as you can 'One, two, three.' Upon hearing the second signal, relax any muscular tensions present." Electrodes in under lip. Interval between vertical time lines in left upper corner = $\frac{1}{25}$ sec.; 1 mm. distance between horizontal lines = 1×10^{-5} volts.

This photograph shows between signal marks three groups of vertical lines, indicating action-potentials, corresponding with the three muscular acts of counting. The microvoltage of the longest lines is about 400.

Fig. 10. *Photographic record of string deflections during imagined counting.* Instruction as in figure 9, excepting that this time the subject is not actually to count, but only to imagine counting, "One, two, three." The amplified galvanometer circuit is here set at higher sensitivity: 1 mm. distance between horizontal lines = 2×10^{-6} volts.

This photograph likewise shows between signal-marks three groups of vertical lines, indicating action-potentials, evidently signifying the occurrence of three muscular acts in imagined counting. The microvoltage of the longest lines is about $\frac{1}{16}$ that of the longest lines in figure 9.

The above results hold, in general, for five subjects upon whom tests were successfully made. A sixth subject, not trained to relax, evidently failed to relax sufficiently when requested to do so; for the string vibrations diminished in magnitude as the minutes passed following the request, but this diminution was not sustained nor did it ever reach approximately zero as with the above-mentioned five subjects. That these continued vibrations were due to action-potentials and not to irregularities in the amplifier circuit was shown by the cessation of vibrations upon short-circuiting the input of the amplifier. In spite of these irregular vibrations during attempted relaxation, a few of the records with this subject clearly show an increase of vibrations following the signal to engage in the mental activity but ceasing soon after the second signal. This evidently is significant since it shows that the occurrence of action-potentials during mental activity involving the speech musculature is not limited to subjects who have been trained to relax; that is, the records of such action-potentials are not artifacts resulting from training. The seventh subject, although she had received considerable training, was under the weight of grave worries and failed to relax sufficiently to give a good base-line for the present tests. Results for these two subjects are not considered further, because, although some records are clearly positive, the readings are generally obscured because of the absence of a straight base-line during attempted relaxation.

For the five subjects who relax well, as shown by a practically quiet string, action-potentials with few exceptions are indicated in the tongue or lip during the mental activities investigated, while during the control tests, the string remains quiet (table 1).

As said above, the control tests with some subjects include the instructions, "Imagine lifting a weight with the right arm" or "Imagine Eiffel Tower in Paris." In previous investigations (Jacobson, 1930a, b, c, d; 1931a, b) it has been found that the foregoing instructions arouse imagination characterized by action-potentials in muscles of the right arm, right arm and ocular regions or ocular regions alone. Our present finding that the tongue and lips characteristically are not active during these control tests again confirms the view that specific muscles contract during each mental process.

During the period of imagination, there occasionally appear one or more series of action-potentials approximately equal in microvoltage to those which appear during muscular movements visible to the naked eye. On the evidence previously considered (1930d), it is here assumed that action-potentials in electrodes connected in muscle tissue signify the occurrence of contraction of fibers. We conclude that while the muscular contractions occurring as a specific part of the physiologic process during imagination are generally microscopic, they are at times grossly visible.

Instructions have been so selected as to include various types of abstract thinking, as well as imagination and recollection of concrete matters. Three of the five subjects, as a part of their training in relaxation, received instruction and devoted many hours of practise at observing the muscle-sense in the speech apparatus during mental activities. After the present tests were made, their reports were secured in detail about each mental act: They agreed that during mental activities involving words or numbers, they generally felt tenseness in the tongue and lips as in saying those words or numbers.[1] These reports conform with our objective findings.

CONCLUSIONS

1. Electrical tests can be satisfactorily carried out for minute contractions in the musculature of speech *a*, if the subject has been trained to relax, and *b*, if the apparatus is sufficiently sensitive and free from interference.

2. During imagination, recollection and concrete or abstract thinking involving words or numbers, muscular contractions characteristically appear as specific components of the physiologic process of mental activity. These contractions generally are minute but sometimes are grossly visible. They occur in at least some of the muscles which participate when the same words or numbers are actually whispered or uttered aloud.

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[1] When the string has been quiet, following the instruction to relax, the subject, if interrupted, reports that the tongue and lips have been relaxed and that mental activities involving words or numbers have been absent. (Whether visualization of words or numbers can occur in some persons with no attendant contractions in the speech musculature is not here tested.)

DIET AND TISSUE GROWTH

VIII. Influence of Vitamins B, G and Undifferentiated B on the Effects Produced by Protein-Rich Diets upon the Kidney of the Rat[1]

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There has long been an association stated or assumed, between dietaries rich in protein and renal damage. It has been observed that when a sufficiently long experimental period (1), (2), (3) was employed, or with certain types of protein (4), there occur pathological lesions as well as increases in size in the kidneys of rats which consumed rations rich in protein. The etiology and prevention of such conditions has interested many investigators, among whom Reader and Drummond (5) claim that renal hypertrophy does not occur in rats maintained on a high protein regimen if the supplements of vitamin B (old nomenclature) are sufficiently increased. Hartwell (6) develops this hypothesis further by stating that yeast or its heat stable moiety prevents enlargement of the kidneys. However, one is left in doubt concerning the efficacy of these vitamins, since Reader and Drummond studied only a few rats and Hartwell's animals had free access to their feces. Enlargement of the kidneys thus becomes a subject of more than academic interest; indeed, the discovery of causes of renal hypertrophy or factors which would prevent such a condition would doubtless throw light on the etiology of associated renal lesions.

The present investigation was undertaken to test the alleged efficacy of vitamins of the B group as factors capable of reducing or preventing the enlargement of the kidneys of rats ingesting high protein rations.

[1] A preliminary report of part of this investigation appears in Proc. Soc. Expt. Biol. Med., 1929, xxvi, 725.

The data presented are taken from the dissertation submitted by Lillias D. Francis to the Graduate School of Yale University in June, 1930, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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EXPERIMENTAL PROCEDURE. *Plan.* Most of the animals (female albino rats) used were subjected to right unilateral nephrectomy at the beginning of the experiment (30 days of age). Any changes which might result from the regimen employed were thus intensified in the remaining kidney. Four groups were left intact, however, to duplicate Reader and Drummond's method more closely and for assurance that the results obtained from rats with one kidney could be duplicated in kind, if not in intensity, in rats having two kidneys. The experimental period lasted 56 days.

Food. Diets containing 18 and 90 per cent casein (table 1) were fed *ad libitum* and vitamin-bearing adjuvants were supplied apart from the ration six times a week. Each rat received a daily allowance of four drops of cod liver oil (116 mgm.) and two drops of wheat germ oil[2] (43 to 45 mgm.) Dried yeast[3] was supplied to some groups at different daily levels of 400, 800 and 1600 mgm. Diets supplemented with these amounts are desig-

TABLE 1

Composition of experimental diets

	DIET A PART OF DIET	DIET B PART OF DIET
	per cent	*per cent*
Casein*	18	90
Dextrin	76	4
Agar**	2	2
Salt mixture	4	4

* Casein no. 453—Casein Mfg. Co., Bainbridge, N. Y.

** Agar Agar, coarse powder—Merck & Co., New York.

*** Osborne, T. B. and L. B. Mendel, Journ. Biol. Chem., 1919 xxxvii, 557.

nated A1, B1; A2, B2; A3, B3; respectively (table 2). The vitamin B in the diet of additional groups was increased by fortifying the allowance of 1.6 gram yeast with tikitiki extract[4] (diets A4, B4) and the vitamin G of other groups, with autoclaved yeast (diets A5, B5).

To test more extensively the effect of either one of the factors, vitamin B or G, as the possible agent which might prevent renal enlargement, each vitamin was fed separately, without yeast, combined with as little of the other factor as previously had been found to be compatible with the well-being of the animals. Diets A6 and B6 supplied an excess of vitamin B and a minimal amount of vitamin G, while rations A7 and B7 furnished a surplus of vitamin B and only a small necessary allowance of G.

[2] Furnished through the courtesy of Mr. M. W. Tapley of E. R. Squibb & Sons, Brooklyn, N. Y. Wheat germ oil no. 665 AH.

[3] Northwestern Yeast Company, Chicago.

[4] Furnished through the courtesy of Doctor Brown, Philippine Bureau of Science, Manila, P. I.

METHOD. At autopsy hearts and kidneys were weighed in the fresh state. Histological sections were made later of several kidneys belonging to each group of animals.

The weight of the heart, of the body and the length of the body has each been referred separately to corresponding Donaldson (7) tables, from which "normal" weights of kidneys have been obtained. The difference between the "normal" and the actual weight of a kidney is interpreted as representing the extent of increase in renal tissue. To avoid using a Donaldson standard, a ratio representing $\frac{\text{kidney weight}}{\text{heart weight}}$ has been employed. Increase in the size of a kidney can therefore be expressed as the difference between two ratios, one for an experimental rat and the other for its control. Results obtained by using the body weight or heart weight standard or the kidney to heart ratio are closely comparable. The body length standard yields results which on the whole compare well with the other methods but are more variable. Degrees of renal hypertrophy here reported have been obtained by the use of the heart weight standard only.

RESULTS AND DISCUSSION. Histological examination revealed no evidence of any pathological condition. This is not surprising, however, since young rats were used and were kept only eight weeks on the high protein regimen (1), (2), (3). Moreover, Newburgh and Curtis (4) have shown that the type of protein we used is relatively innocuous in producing pathological conditions in the kidneys of rats.

Enlargement of the kidneys due to ingestion of rations rich in protein is not lessened by added yeast, nor by fortifying a large yeast allowance with additional vitamin B or with extra vitamin G. Neither is the renal hypertrophy reduced by feeding an excess of vitamin B with a minimum of G nor by a surplus of vitamin G accompanying the smallest essential quantity of B. Significant differences ($\frac{\text{D}}{\text{PED}} = 3$ or more) in renal enlargement are associated with increased consumption of protein irrespective of vitamin supplements.

The difference between the increases in the size of the kidneys of control groups and their experimental mates (table 2) represents the degree of renal enlargement due to dissimilarity in the diets. The sequence of diets in table 2 is determined by that of the ascending order of increases in renal tissue. There is a striking variation in the order of the B diets from that of the A diets. If vitamins B, G and undifferentiated B play any rôle in preventing or reducing hypertrophy of the kidneys, we should expect the sequence of these vitamin supplements to the B diet to conform with those of the A ration. But such is not the case.

Furthermore, if ingestion of a large excess of yeast could prevent or even materially lessen the increase in the size of the kidneys which results when

TABLE 2

Renal enlargement correlated with total protein ingested by rats fed diets containing 18 (A) and 90 per cent (B) casein, from 30 to 86 days of age

INCREASE IN SIZE OF KIDNEY—AVERAGE	SIGNIFICANCE RATIO $\frac{D}{PEd}$	DIFFERENCE IN RENAL ENLARGEMENT BETWEEN GROUPS FED DIETS A AND B	PROTEIN INGESTED* AVERAGE CALORIES	SIGNIFICANCE RATIO $\frac{D}{PEd}$	DIETS**	NUMBER OF RATS IN EACH GROUP
Nephrectomized rats						
per cent	Cf. A7	*per cent*		Cf. A7		
39 ±1.3***			374 ± 4.6***		A7	12
52 ±2.7	4.3		454 ± 7.4	9.2	A2	14
53 ±2.6	4.8		496 ± 8.7	12.2	A5	13
56 ±4.9	3.3		350 ± 9.7	2.3	A6	12
57 ±2.9	5.7		399 ±11.5	2.0	A1	14
60 ±3.2	6.4		527 ± 1.4	31.8	A4	12
64 ±2.9	7.8		480 ± 7.1	12.5	A3	21
	Cf. B1			Cf. B1		
115 ±4.0		58	1413 ±46.0		B1	13
119 ±4.3	0.7	67	1493 ±43.2	1.3	B2	14
127 ±4.9	1.6	67	1672 ±42.3	4.2	B4	12
127 ±4.3	2.0	71	1187 ±33.5	4.0	B6	12
127 ±4.2	2.1	74	1690 ±43.3	4.4	B5	12
134 ±6.8	2.4	70	1465 ±45.8	0.8	B3	13
148 ±3.3	6.3	109	1519 ±32.7	1.9	B7	12
Intact rats						
	Cf. A1			Cf. A1		
−7 ±1.7			466 ± 8.4		A1	12
4 ±1.3	5.1		508 ± 9.3	3.4	A3	13
	Cf. B1			Cf. B1		
39 ±2.7		46	1651 ±32.4		B1	12
50 ±2.6	3.0	46	1686 ±18.3	0.9	B3	12

* Includes nitrogen of yeast calculated as protein.

** Yeast (Y), autoclaved yeast (AY) and tikitiki extract (TT) supplements to the A and B diets:

A1 and B1 = 0.4 gm. Y.
A2 and B2 = 0.8 gm. Y.
A3 and B3 = 1.6 gm. Y.
A4 and B4 = 1.6 gm. Y + 3 drops TT.
A5 and B5 = 1.6 gm. Y + 7 per cent AY reduced to 5 per cent when food intake approximated 10 gm. daily.
A6 and B6 = 0.8 gm. AY + 1 drop TT (30 mgm.) increased slowly to a maximum of 3 drops daily.
A7 and B7 = 5 drops TT increased gradually to a maximum of 12 drops daily. During the last four weeks of the experiment 0.4 gm. AY was supplied twice weekly.

*** Probable error.

rations rich in protein are consumed, we should expect the renal enlargement of the group on the B3 diet to be less, or certainly no more, than that of the group fed the B1 ration. But, again, such is not the case. There is an appreciable difference between the renal enlargement of the groups maintained on the B1 and B3 diets, yet the consumption of protein calories shows a significant ratio of only 0.8 for the nephrectomized and 0.9 for the intact animals. Indeed one might be inclined to conclude that added yeast increases rather than decreases the enlargement of the kidneys of rats maintained on high concentrations of casein.

With such lack of uniformity in dietary sequence as table 2 shows and an increased renal enlargement accompanying an ingestion of augmented yeast supplements, our findings are at variance with the hypothesis of the English investigators (5), (6). Evidence is thus afforded that neither the amount nor the combinations of vitamins B, G and undifferentiated B lessen or prevent the enlargement of the kidneys of rats consuming rations rich in protein.

SUMMARY

Eighteen groups of rats were maintained from 30 to 86 days of age on diets containing 18 and 90 per cent casein supplemented with necessary vitamins.

Histological examination revealed the presence of no pathological renal lesions.

Renal enlargement due to the high-protein ration occurred in intact rats as well as in those having one kidney.

This increase in the size of the kidneys was neither lessened nor prevented by the use of any amount or combination of vitamins B, G and undifferentiated B employed.

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THE POSTERIOR PITUITARY HORMONE IN METABOLISM

I. The Effect of Pitressin upon the Carbohydrate Reserves of the Normal Rabbit

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The rôle of the posterior pituitary hormone in carbohydrate metabolism is at present more obscure than are the rôles of insulin and adrenalin. That the hormone affects carbohydrate metabolism is generally admitted. Most workers agree that in the normal animal hyperglycemia is produced. (Partos and Klatz-Klein, 1921; Burn, 1923; Tingle and Imbrie, 1926; Bacq and Dworkin, 1930.) Since pituitrin lessens the effect of exogenous insulin (Burn, 1923) and hypophysectomized animals become markedly sensitive to insulin (Geiling, Campbell and Ishikawa, 1927), the hormones are regarded as antagonistic in action. Strangely enough, pituitrin is also apparently antagonistic to adrenalin (Laurin, 1917), as far as the hyperglycemia phenomenon is concerned. In the case of a glucose tolerance hyperglycemia (Tingle and Imbrie, 1926), the effect of pituitrin is first retarding, then augmenting. It has been suggested that pituitrin plays the rôle of a blood sugar regulating hormone, raising the sugar when low, lowering it when high. No attempt will be made to catalogue all the observations reported in regard to pituitrin and carbohydrate metabolism. A brief summary is given in Laquer's *Hormone und Innere Sekretions* and an excellent discussion by Geiling *et al.*

It is apparent that in the past attention has been focused on the blood. The effect upon the carbohydrate reserves, hepatic or peripheral, has not been studied. The relation between liver glycogen, blood sugar, muscle sugar, muscle glycogen, and lactic acid has not been established.

Object. In the present paper our studies have been confined to the normal rabbit. Pitressin instead of pituitrin has been used. According to Abel (1930) pitressin may be an *abbau* product of the complex pituitary hormone. Its use eliminates any complicating effects which might be ascribed to the oxytocic principle. However, since Gruber (1929) found differences in the action of pitressin and pituitary extract upon the blood pressure of unanesthetized dogs, our choice may be questioned. This

author does not consider the "Pressor Assay Method" which standardizes the pitressin an index of the true activity. The plan of experimentation with pitressin was as follows:

1. Establishment of the lethal dose and the pharmaceutical and physiological range.

2. Comparison of effects of intravenous, subcutaneous, and continous intravenous injections upon blood sugar and lactic acid.

3. Determination of blood urea nitrogen, amino acid nitrogen, inorganic phosphorus, and cholesterol, constituents which might reflect changes in protein or fat metabolism.

4. Determination of blood volume changes by following the oxygen capacity.

5. Comparison of the carbohydrate reserve in dosed and control animals.

Studies on medullary adrenalectomized and guanidine deglycogenated animals will appear in later papers.

LETHAL DOSE. *Intravenous injection.* Although 0.2 dog unit per kilo of pitressin given intravenously to a rabbit under amytal anesthesia will produce a rise in blood pressure, over one hundred times the dose of pitressin may be given intravenously in a single dose to unanesthetized animals without lethal effect. Six rabbits were given respectively single intravenous doses of pitressin. The doses per kilo were 4, 9, 15, 20, 24, and 25 dog units. The rabbit which received the highest dose died three hours after the injection. All the others survived. In the case of adrenalin the range between the minimum blood pressure raising dose and the minimum lethal dose is approximately the same, 0.03 to 0.06 mgm. per kilo given intravenously being lethal to the rabbit, while 0.0008 to 0.00010 mgm. per kilo minute is the smallest amount bringing about a rise in blood pressure. In the case of adrenalin, the dose which is lethal as a single injection may be given with impunity as a continuous uniform intravenous injection over an extended period of time. With pitressin the situation was found to be reversed. Six rabbits were given continuous intravenous injections of pitressin at the rates of 2.6, 3.1, 7.9, 11.3, 12.2, and 15 dog units per kilo hour. Two rabbits died the second and third hours of injection respectively on doses of 3.1 and 15.0 dog units per kilo hour.

Subcutaneous injection. One rabbit, which died 24 hours after receiving 15 units of pitressin per kilo subcutaneously, was prostrated immediately after dosing. No change in blood sugar was noted the first hour.

BLOOD SUGAR. *Continuous intravenous injection.* Doses ranging from 2.6 to 15.0 dog units per kilo hour were injected into rabbits for periods of time ranging from one to four hours. The lowest dose failed to produce hyperglycemia. Rises in blood sugar from 115 to 248, from 115 to 198, from 110 to 151, and from 91 to 145 were produced by doses ranging from 3.1 to 12.2 dog units per kilo hour. The blood sugar curve did not con-

tinue to rise or even persist at a maximum level, but steadily fell, even as early as the second hour, when the continuous intravenous injection was continued over three or four hours. In the case of adrenalin, the peak of the curve was usually at the third hour, when the descent was quite gradual. In one instance, in which the animal died the third hour, 15 dog units of pitressin per kilo hour failed to produce a hyperglycemia the second hour. No data were obtained for the intervening period.

Intravenous injection. Six experiments were performed giving single intravenous doses of pitressin. Doses of 4 and 9 dog units per kilo produced a maximum hyperglycemia one-fourth hour after dosing, changes of 125 to 182, and of 78 to 156 in the blood sugar being produced. Twenty dog units per kilo raised the blood sugar from 88 to 162 in one-half hour. In two experiments in which 15 and 25 dog units per kilo were given respectively no significant change in blood sugar was observed until the fourth hour. In one experiment, 24 dog units per kilo raised the blood sugar from 96 to 198 in one-half hour. At three and one-half hours the blood sugar had risen to 254, when the animal died. The results are by no means uniform.

Subcutaneous injection. Ten experiments were performed with subcutaneous injections. Eight and 12 dog units per kilo produced a significant hyperglycemia one-half to one hour after dosing, with a rapid fall in blood sugar to normal in the following period. Fifteen dog units per kilo in three instances produced no rise in blood sugar the first hour. In five other instances a significant rise was produced.

Lactic acid. In studying blood lactic acid curves in the rabbit it is very important that the animal be in a resting state, as any muscular activity will raise the blood lactic acid, a few kicks sending it from a basal value below 20 mgm. per 100 cc. to over 200 mgm. per 100 cc. In bleeding rabbits we have in the past been in the habit of placing their necks in a yoke. Basal lactic acid values obtained by us (Bischoff and Long, 1930) in the past have been somewhat higher than those reported by Cori and Cori. Following a suggestion of these workers (Cori, Cori, and Buchwald, 1930), the rabbits have been placed in a box, the bleeding being done without any attempt to confine the rabbit in any position. When the box is just large enough to hold the rabbit the rabbit will usually remain in a state of rest, even for hours. In the present series of experiments no lactic acid values are reported if the rabbit made any attempt to leave his position. Resting lactic acid values comparable to those of Cori and Cori were obtained. We are convinced that by confining the rabbit by a yoke he offers resistance to the yoke without actually kicking, so that the blood lactic acid may rise over 100 mgm. per 100 cc. In continuous intravenous injection experiments the injection needle was held in place in the marginal ear vein with adhesive tape. It is quite remarkable that

rabbits will make no attempt to break away for periods as long as seven hours. Occasionally an obstreperous animal will be found which cannot be used for work on lactic acid.

Continuous intravenous injection. Increases in the blood lactic acid appreciably above the basal range, which in the present series varied from 10 to 30 mgm. per 100 cc., were observed in all cases studied, with the exception of the minimal dose of 2.6 dog units pitressin per kilo hour. The lactic acid curve did not follow the blood sugar curve in all cases. Fifteen dog units per kilo hour produced a rise in lactic acid from 25 to 69 the second hour with no change in blood sugar. On the other hand, 7.9 dog units per kilo hour in one rabbit raised the blood sugar from 110 to 151 the second hour without affecting the lactic acid. At death, preceded by convulsions, the blood lactic acid values of two rabbits were high, one showing a normal blood sugar.

Intravenous injection. Lactic acid curves were followed in two rabbits, which were given 9 and 25 dog units per kilo of pitressin in a single intravenous injection. In both cases an appreciable rise in lactic acid was observed the first hour. In one of these experiments the blood sugar remained normal at the time the lactic acid was high. A hyperglycemia was observed the fifth hour, when the lactic acid had returned to normal.

Subcutaneous injection. Lactic acid curves were followed in two experiments for rabbits given pitressin subcutaneously in doses of 12 and 15 dog units per kilo. A slight rise in lactic acid without change in blood sugar was noted for the higher dose. A considerable rise in lactic acid, with a curve more delayed and more persisting than the blood sugar curve, was observed for the other rabbit.

Miscellaneous blood constituents. The blood amino acid nitrogen curve was determined for a rabbit which had received a subcutaneous injection of 12 dog units of pitressin per kilo and one which had received a single intravenous dose of 9 dog units per kilo. A slight rise, which was, however, within normal limits, was observed in both cases. As the amino acid nitrogen value has a tendency to fall on successive bleedings in the rabbit (Bischoff and Long, 1929), the result may be significant. The blood urea nitrogen showed no significant change following the subcutaneous injection. No other determinations were made. Analyses for blood cholesterol were made in three experiments, one following the subcutaneous injection of 15 dog units per kilo of pitressin, one following the single intravenous injection of 25 dog units per kilo, and one following the continuous intravenous injection of 15.0 dog units per kilo hour for three hours. All the values obtained were within the normal range for the rabbit.

As the changes in blood sugar are regarded by some workers as being masked due to changes in blood volume following the action of pituitrin (Partos and Klatz-Klein, 1921), the oxygen capacity of the blood was

determined for five intervals following the single intravenous injection of 20 dog units of pitressin per kilo. Since the oxygen capacity is the most accurate method for determining the hemoglobin, and as this substance would hardly be expected to change in the course of the experiment, any significant changes in the hydration of the blood should be noted. In the experiment recorded, a 4.2 kilo rabbit was used. During the course of the experiment 30 cc. of blood were drawn for analytical purposes. The oxygen capacity fell from 19.9 to 18.0 per cent. The fall in capacity would indicate a volume increase of 10 per cent. The experiment was duplicated, giving pitressin subcutaneously with similar results. An increase in blood volume amounting to 6 and 8 per cent was observed the first half-hour following the injections of 20 units of pitressin per kilo. The amount of blood necessary for one analysis is less than 2 per cent of the total blood volume. A single bleeding could hardly account for the change observed following pitressin on the basis of loss of blood. An increase of 10 per cent in hydration would in no case affect appreciably the other blood constituents. In one experiment the moisture content of dosed and control muscle was determined five hours after the subcutaneous injection of 15 dog units of pitressin per kilo. The control muscle analyzed 24.1 per cent solids, the muscle of the dosed rabbit 22.9 per cent. It would be hazardous to make any deductions without knowing the normal variation. The figures do not indicate a shift of water from the muscles to the blood. Partos and Klatz-Klein (1921), on the basis of blood solid determinations, found a maximum variation of 33 per cent in blood hydration after the action of pituitrin.

GLYCOGEN RESERVES. Muscle and liver glycogen determinations were made in five experiments following a continuous intravenous injection of adrenalin. The time of injection varied from one to four hours. The animals were killed from two to five hours after the injection was begun. The technique for determining liver and muscle glycogen has been described in former papers (Bischoff and Long, 1930). In the present series a control litter mate, fasted the same length of time, was killed for glycogen determination at the time the dosed rabbit was killed. Muscle sugars were determined in three series. Glycogen and muscle sugar values were also determined five hours after the subcutaneous injection of pitressin. In this experiment 15 dog units per kilo were given. In the continuous intravenous injection series the total amount of pitressin perfused per kilo ranged from 10.3 to 45 dog units.

Barring the rabbit which was moribund at the third hour, when the samples for analysis were taken, there is no significant consistent change in the glycogen reserves indicated between dosed and control rabbits. The animal which was dying had been given a total of 45 dog units per kilo during a three-hour continuous intravenous injection. A muscle glyco-

Effect of continuous intravenous injections of pitressin upon the blood and tissue constituents

	DOSE PER KILOGRAM IN DOG UNITS	INJECTION RATE PER HOUR	INJECTION TIME IN HOURS	BLOOD FINDINGS IN MGM. PER 100 CC.								TISSUE FINDINGS IN MGM. PER 100 gm.		
				Constituent	Initial	0.5 hour	1.0 hour	1.5 hours	2.0 hours	3.0 hours	4.0 hours	5.0 hours	Liver glycogen	Muscle glycogen	Muscle sugar
Dosed	10.0	7.2	0.3	Sugar	115	190	212	240	248				871	223	166 (293)
		11.3	1.0	Lactic acid	19	75	175	200	225						
				Inorganic P	5.9	6.5	6.5	5.7	5.3						
Control													482	374	80 (222)
Dosed	6.3	3.1	2.0	Sugar	115		198		156*						
				Lactic acid			62		290*						
				Inorganic P	9.1		8.4		10.0*						
Dosed	10.3	2.6	4.0	Sugar	92		109		85	82	100	106	1,470	112	
				Lactic acid			18		15	14	16	25			
Control													1,182	285	
Dosed	31.5	7.9	4.0	Sugar	110				151		132	141	1,267	280	88
				Lactic acid	30				18		25	40			
Control													1,080	260	98
Dosed	24.4	12.2	2.0	Sugar	91				145	96	100	87	154	181	79
				Lactic acid	15				35	17		17			
Control													442	295	80
Dosed	45.0	15.0	3.0	Sugar	71				84	103*			110	69	
				Lactic acid	25				69	132*					
				Cholesterol					68	65*					

Figures in parentheses indicate muscle sugar after hydrolysis.

* Convulsions, death.

Effect of single intravenous and subcutaneous injections of pitressin upon the blood constituents

DOSE PER KILO DOG UNITS	MODE OF INJECTION	CONSTITUENTS	INITIAL	0.25 HOUR	0.5 HOUR	0.75 HOUR	1.0 HOUR	1.5 HOURS	2.0 HOURS	2.5 HOURS	3.0 HOURS	3.5 HOURS	4.0 HOURS	5.0 HOURS
4	Intravenous		125	182	178	156	141							
9	Intravenous	Sugar	78	156		143	111							
		Lactic acid	22	160		121	37							
		Amino acid N	9.1	9.5			10.6							
15	Intravenous	Sugar	97				112		129			127	163	
20	Intravenous	Sugar	88		162		129		96		97			
		THb	19.9		18.7		18.4		18.3		18.0			
24	Intravenous	Sugar	96		198		176		285			254		
		Cholesterol	88		85		100					*		
25	Intravenous	Sugar	91	100	108		106	133	138	136	127		154	
		Lactic acid	12	48	55			24		17			15	
		Cholesterol	77								58			
8	Subcutaneous	Sugar	118				200		118		125		125	
12	Subcutaneous	Sugar	92		141		132	114	112					
		Lactic acid	14		111		165	67	65					
		Amino acid N	8.4		10.8		10.8	11.2	11.2					
		Urea N	24.7		21.0			25.4	23.0					
15	Subcutaneous	Sugar	94		103		92							
15	Subcutaneous	Sugar	80		90		92							*
15	Subcutaneous	Sugar	85		111		135							
15	Subcutaneous	Sugar	103	108	103	114			121					
15	Subcutaneous	Sugar	118	141	135	143			148					
15	Subcutaneous	Sugar	97	118	123	160			125					
15	Subcutaneous	Sugar	97				95		103		94		91	96†
		Lactic acid	10.5				20.3				14.3		10.8	11.9
		Cholesterol	74						76		74		70	70

* Dead.

† The animal was killed the fifth hour, the liver glycogen being 684, the muscle glycogen, 232 mgm. per 100 gm.

gen value of 69 and a liver glycogen value of 110 indicated deglycogenation. This animal passed through a series of convulsions before death. In the other five experiments, the muscle glycogen was lower than the control muscle glycogen in three cases and higher in two cases. The liver glycogen values for the dosed animals were higher in four and lower in one case. However, there was a considerably greater difference in the liver glycogen in different series between either dosed or control animals than there was between a dosed and a control litter mate of the same experiment. On the basis of Goldblatt (1929) and Sahyun and Luck's (1929) observations, these differences would be expected, since our different series were not of the same age. These authors have pointed out that marked differences in the glycogen content of tissues exist in the rabbit at different ages.

In a former paper, the muscle glycogen values for control rabbits fasted 24 to 29 hours were determined in exactly the same manner as the glycogen determination in the present series. Using the seven values of the former paper and the five control values of the present paper, the mean for the muscle glycogen is 282, the standard deviation of the mean being $\pm$ 28. Assuming that the accuracy of establishing the hypothetical mean varies as the square root of the number of samples, the standard deviation of the mean for the five control glycogens would be 43. Using the five control values, the standard deviation of the mean varies between 51 and 55, depending upon whether n^2 or $n(n-1)$ is used. At any rate the difference of the means for the dosed and control muscle glycogens is $272-206 = 66$, so that the difference lies between once and twice the standard deviation of the mean, whatever basis of computation is used. *It would be safe to conclude that even large doses of pitressin approaching the lethal dose have only a slight effect, if any, in lowering the muscle glycogen. If pitressin has any effect upon the liver glycogen it is to bring about a slight increase.* In the case of the liver, the tremendous variation in the normal glycogen content makes dangerous any deduction (with the exception of virtual depletion, as in the first hour of adrenalin activity) in regard to small changes.

Muscle sugar. In four experiments the muscle sugar of the pitressin-dosed rabbit was determined along with the muscle and liver glycogen. The muscle sugars of control litter mates were also determined. In two instances the blood sugars at the time of killing were normal. Resting muscle sugar values were obtained. In the two other experiments the blood sugars were hyperglycemic, at the time of killing, 248 and 141 respectively. Muscle sugar values of 166 and 88 against control values of 80 and 98 were obtained. The high muscle sugar values were obtained two hours after the initial dosing of pitressin, the normal values five hours after the initial dosing.

Discussion. The results of our experiments show that there is a far greater individual variation in animals in their response to pitressin than

there is to adrenalin and insulin. The dose lethal to one animal will be without effect upon the blood sugar and lactic acid in another. Fifteen dog units per kilo subcutaneously were lethal in one case. In three animals this dose did not even produce a slight hyperglycemia. Six and three-tenths units per kilo given as a continuous intravenous injection over two hours was fatal, producing a decided hyperglycemia. On the other hand, 10.3 units per kilo given over four hours (practically the same rate) produced no effect whatsoever. *These results indicate that the minimum dose of pitressin which affects carbohydrate metabolism in the rabbit is a pharmaceutical dose.* Bacq and Dworkin (1930), giving two to four dog units in a single intravenous injection to the cat, observed rises in blood sugar amounting to 150 to 200 mgm. per 100 cc. in some cases at 15 minutes. They consider the duration of the hyperglycemia as comparatively long lasting. Nitzescu and Benetato (1930), giving five units per kilo to the rabbit, observed a 100 mgm. rise the first thirty minutes, practically normal values being observed at one hour. It is evident from these experiments that the rabbit is less sensitive than the cat in the production of pitressin hyperglycemia. The hyperglycemia curves of Nitzescu and Benetato for single intravenous injections of pitressin are very much like those obtained by Cori and Cori for adrenalin. The effect is very transient and any measurable effect upon the carbohydrate reserves could hardly be expected. The hyperglycemia curves for subcutaneous injections observed by Nitzescu and Benetato confirm those obtained by us for certain animals. These authors did not observe as many negative responses as did we. In the case of adrenalin it is now generally conceded that profound changes in carbohydrate metabolism may be brought about by physiological doses, the crucial test having been to establish that doses which did not raise the blood pressure affected the blood sugar and lactic acid. In the case of pitressin this criterion cannot be applied, since pitressin does not raise the blood pressure of unanesthetized animals (Gruber, 1929). Since a continuous intravenous dose of pitressin may be more toxic than a single intravenous dose, some other factor than arterial spasm is concerned in the production of death. The great variation in response of individual animals to pituitary extracts has been attributed to conditions of hypo- and hyper-pituitarism in so-called normal animals, so that a basis of comparison of the results upon two different animals is not considered justified. While this criticism might be valid in regard to the production of such changes as hyperglycemia, it could hardly be applied to lethal effects.

Certain effects of pitressin, when they occur, resemble almost exactly the effects produced by adrenalin, notably the rise in blood pressure, blood sugar, and blood lactic acid. In fact one is tempted to account for them on the basis that pitressin stimulates the release of adrenalin and does not itself produce the reactions. The explanation is strengthened by the ob-

servation that the effect may or may not occur, that is, that adrenalin may not always be liberated. There is likely to be more individual variation when a hormone produces an effect through the action of another hormone rather than when it functions directly to produce the effect. Evidence opposing this explanation is the inhibitory effect which pituitrin is supposed to have upon adrenalin hyperglycemia or upon a glucose tolerance blood sugar curve. The anomalous effects upon blood pressure are likewise not characteristic of adrenalin. The delayed blood sugar curve following the action of pitressin during a glucose tolerance may be explained by the heart-slowing action of pitressin, which would retard the distribution of glucose on a purely mechanical basis if the blood flow is also retarded, and account for the delayed curve. A change in the rate of blood flow could conceivably also decrease the blood sugar curve following the action of adrenalin subcutaneously administered. In studying the effect of pitressin upon the tissues we have been unable to demonstrate any appreciable changes in the glycogen reserves, so that if pitressin functions by stimulating adrenalin, the amount of adrenalin liberated is minute and transient. The question now arises as to whether the maximum hyperglycemic effects produced by pitressin could have taken place through the liberation of adrenalin without changing the glycogen reserves. Data for considering this question are available from our work on the continuous intravenous injections of adrenalin, although it must be remembered that the blood sugar level gives little information as to the state of the carbohydrate reserves in the hepatic and peripheral tissues. The two highest muscle glycogen values obtained in the adrenalin series were 131 and 104 mgm. per 100 grams respectively, the lowest control being 111. Doses of 0.04 and 0.10 mgm. adrenalin given by continous intravenous injection were used in these two experiments, the blood sugar rising to 220 and 254 respectively. Only in two of over twenty pitressin experiments did the blood sugar reach these heights. In one of these experiments the lethal dose of pitressin was given. On the other hand, adrenalin may deglycogenate in some animals with a relatively low blood sugar curve, so that the blood sugar curves following pitressin are sufficiently high to be produced by deglycogenating doses of adrenalin, which we know is not the case. Judging by the blood sugar values, the mild hyperglycemia and lactic acidemia produced by pitressin could be accounted for by the liberation of a small subdeglycogenating amount of adrenalin, but the range of the hyperglycemia is not below that which occasionally may be observed with deglycogenating doses of adrenalin. Since pitressin masks the blood sugar values by slight changes in blood volume, and since the blood flow is possibly affected, blood values cannot be taken as crucial evidence in settling the problem. A mechanism which is possible but which would, with our present incomplete knowledge, appear unlikely, is that the liberation of glucose

from the liver is compensated for by the liberation of lactic acid from the muscles, so that the glycogen reserves in both liver and muscle are replenished as fast as they are depleted. In order to settle the question, studies with medullary adrenalectomized animals are necessary. The studies of the present paper have added to rather than diminished the obscurity which clouds any understanding of the physiological activity of the post pituitary hormone. Several questions capable of experimental investigation have been raised: 1, the effect of pitressin upon medullary adrenalectomized animals, and 2, the effect upon the rate of blood flow. 3. The observation that pitressin may produce a lactic acidemia without affecting the peripheral glycogen reserve suggests further investigation of fat metabolism.

SUMMARY

1. The production of hyperlacticacidemia is as characteristic of pitressin as the production of hyperglycemia.

2. Doses of pitressin which are effective in producing hyperglycemia and lactic acidemia in the rabbit are pharmaceutical and not physiological. They approach the lethal dose. Hyperglycemia and lactic acidemia do not run parallel.

3. The toxic effect of pitressin is not reduced when given as a continuous intravenous injection and when so given may be more pronounced than the effect of a single intravenous dose.

4. Pharmaceutical doses of pitressin, whether given intravenously or subcutaneously, produce no changes in the glycogen reserves of the muscle outside the limits of normal expected variation. A possible but not clearly established increase in liver glycogen takes place. No changes without the normal limit in the blood amino acid nitrogen, urea nitrogen, or cholesterol are produced.

5. As measured by the oxygen capacity, a pharmaceutical dose of pitressin increases the blood volume by ten per cent in the course of three hours.

6. The muscle sugar rises above the resting level during a pitressin hyperglycemia.

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SYNTHESIS OF ADEQUATE PROTEIN IN THE GLANDS OF THE PIGEON CROP[1]

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The use of pigeons in the study of many phases of nutrition offers several advantages over the use of rats, guinea pigs and chickens. Pigeons are able to tolerate well a deficient ration, to live in small quarters for a considerable time and to get along on small amounts of feed. Besides these advantages, their method of feeding their young, "without spilling a drop" is unique and interesting. Their "milk" which is secreted in the crops of both male and female is of especial importance in nutrition studies.

Nutritive value of different cereals. During the years in which experiments with pigeons have been in progress here (1), (2), (3) it has been a matter of no little curiosity as to the reason why young squabs, whose parents had received nothing but corn, minerals and water the previous year, could grow so well while young chickens, from eggs produced on a similar ration, were unable to survive for any considerable period. Since the manner of feeding was the only evident difference between the two cases, it was concluded that the pigeons had received nutrients from their parents which the chickens had lacked. It is well known that most grains when fed alone are somewhat deficient or unbalanced as a ration and that corn is especially deficient in several important amino acids which are believed to be necessary to the animal's welfare.

Value of corn as a feed for rats. The following quotation from McCollum, Simmonds and Pitz (4) emphasizes the deficiencies of corn in the nourishment of rats—"We have attempted several times to nourish young rats with a diet restricted to the germs of the maize kernel, and to mixtures of the maize in several proportions. These attempts have been uniformly unsuccessful."

Osborne and Mendel (5) writing shortly afterwards state, "Our experiments indicate that it ought to be possible to make an animal grow on a

[1] Presented at Biological section of meeting, American Chemical Society, Nashville, Tenn., April 10, 1930.

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diet in which the maize is the sole source of protein, provided a preparation of the total proteins could be obtained which would permit feeding them in sufficient quantity so that enough of these amino acids which are present in certain of the proteins and not the others would be available to meet the nutritive requirements of the organism."

Study of the glands and contents of pigeon crops. It was once thought that the parent birds could work over enough corn to make the growth of the squabs nearly normal. Later this was believed to be impossible and an effort was made to examine the pigeon crops and their contents of various sized birds to see if they were different in any way from the crop of a chicken. Accordingly many pigeons and squabs were killed, their crops cut open, and with their contents were studied chemically by one of the writers. The other investigated histologically the construction and possible glandular nature of the folds making up the crop.

TABLE 1

Showing the composition of the evaporated serum from pigeons fed on corn

CONSTITUENT	PER CENT
Ash	4.5 to 5.5
P_2O_5	2.0 to 2.5
K_2O	0.8
MgO	0.0 to 1.2
CaO	0.5 to 1.5
Carbohydrates	None
Fat (crude)	25.0 to 29.0
Protein (N 6.25)	13.0 to 14.5

TABLE 2

Data by Stevens on analysis of milk from pigeon fed whole yellow corn

CONSTITUENT	PER CENT
Total solids	20.0 to 30.0
Total protein (N 6.25)	14.0 to 16.0
Dry material	"
Ether extract	30.0 to 39.0
P_2O_5	1.9 to 2.7
CaO	1.1 to 1.9
Total ash	5.0 to 6.0
Carbohydrate	None

Enzymes,—rennet, amylases, saccharases.

Analysis of the evaporated pigeon milk. The serum like substance obtained from the crops of squabs was evaporated to dryness under reduced pressure at a temperature of 60°C. The solid matter obtained varied from 18 to 25 per cent in squabs from corn fed parents and from 14 to 20 per cent in those from soy beans. Pigeons receiving the beans used much more water each day than those on any other feed. The approximate composition of this dried resinous solid from the serum of a corn fed pigeon is given in table 1.

The writers learned recently that an unpublished study of the composition of pigeon milk had been made by Henry Stevens (6). He has kindly sent the following report of his analysis of the serum from the crops of living birds.

In addition to the analysis of this solid just given, another reference on

pigeon milk reported by Schäffer (7) in an old physiology is as follows: "The so-called pigeon milk with which the young birds are fed by both parents during the earlier days of life is practically a sebaceous secretion of temporary glands formed in the lateral pouches of the crop in both cock and hen. It contains fat, a proteid clotting with rennet, globulin, salts, and water but is free from sugar."

Histological studies on the glands in the crop. The histological studies on the various parts of the crop of a pigeon revealed the presence of glands which open out into the crop and furnish the answer for the presence of the milk serum. These glands are absent from the crop of a chicken. The following description shows the difference between the crops of pigeons and chickens.

Fig. 1

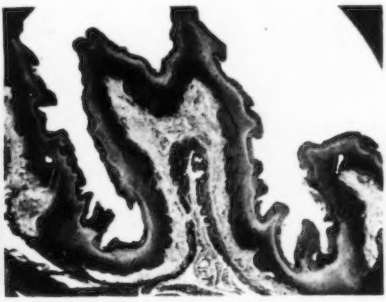

Fig. 2

Fig. 1. Showing the secreting glands in folds contained in a pigeon crop. These glands deliver the "pigeon milk" into the crop.

Fig. 2. Showing the folds present in a chicken crop similar to those in a pigeon, but they contain no milk glands.

The crop of a pigeon is lined by stratified squamous epithelium, which rests on a rather dense areolar tissue the superficial portion of which contains many elastic fibers. At frequent intervals the connective tissue is prolonged into the epithelium in the form of papillae, similar to those of the human skin.

The submucosa consists of a rather loose areolar tissue containing blood vessels and lymphatics.

The muscular tissue is arranged in two layers, an inner longitudinal and an outer circular layer.

In the lower end of the crop and extending into the esophagus are found many branched alveolar glands whose ducts open upon the inner surface of the crop and esophagus. Glands are formed by hypertrophy of the basal cells of the lining epithelium and these cells are desquamated

during secretion. The glandular epithelium rests directly upon the connective tissue which supports the glands. The duct of each gland is lined with epithelium which is continuous with that of the stratified squamous epithelium.

The crop of a chicken differs from that of a pigeon in that its lining epithelium contains many more layers of cells, the outermost layers of which are cornified, and there are no glands present in the crop or esophagus.

These differences may be easily noted by comparing figures 1 and 2. Figure 1, a portion of the crop of a pigeon, shows the glands opening out into the crop. Figure 2, the crop of a chicken, shows the absence of any milk glands.

A more recent search of the literature on this subject revealed some work on the glandular structure of a pigeon crop by Dulzetto (8). His histological findings were somewhat similar to those noted above.

TABLE 3

Relation of grain fed to weekly gain in squab weight

FEED	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK
	grams	*grams*	*grams*	*grams*	*grams*
Hemp	80	194	210	240	350
Kaffer	78	154	178	220	280
Corn	48	96	140	166	180
Wheat	74	186	220	280	310
Soy beans	80	164	210	225	290
Oats (hulled)	70	168	320	355	380

Growth rate of squabs fed on different grains. The rate of growth of squabs fed on single grains and minerals was less rapid than that of those fed a normal ration. Yet they were able to make a constant increase in weight as long as the parent birds continued to feed them which was usually from one and a half to two months. After this feeding period was over the increase in weight of the corn fed birds was very slow. This slow growth emphasized the food value of the pigeon milk over that of the corn and minerals used after weaning. Also there is evidence to indicate that the milk glands in the crops of the parents only function actively during the feeding period. Table 3 gives the average weekly gains of squabs fed on different grains.

It is difficult to get the correct increase in weight of squabs with the usual two birds to a nest as one of them is usually more persistent in its coaxing for food and so receives more than the other. Hence it is not uncommon for one of the squabs to weigh twice as much as the other at the

end of three or four weeks. Hemp seems to be a remarkably well balanced grain and all squabs receiving this grain made good gains both before and after weaning. Corn squabs made slow progress even in the egg as well as while being fed. After the weaning it is usually only a bare existence, but now and then a bird gets such a good start while being fed that it continues to gain in weight even after weaning and finally reaches a nearly normal size.

SUMMARY

Both male and female pigeons contain glands in their crops which secrete a milk during the period they are feeding their young. This fluid is high in fat, protein, mineral matter and contains such enzymes as rennet, amylases and saccharases, etc., all of which aid in providing a suitable food for the nourishment of the young pigeons. These glands are not present in the crops of chickens hence they have no additional source of food with which to nourish their young and the young chickens cannot live on corn as the squabs do.

It is suggested that a possible synthesis may go on in the glands of the pigeon's crop to supplement the amino acids known to be missing in the corn and necessary for maintenance and growth.

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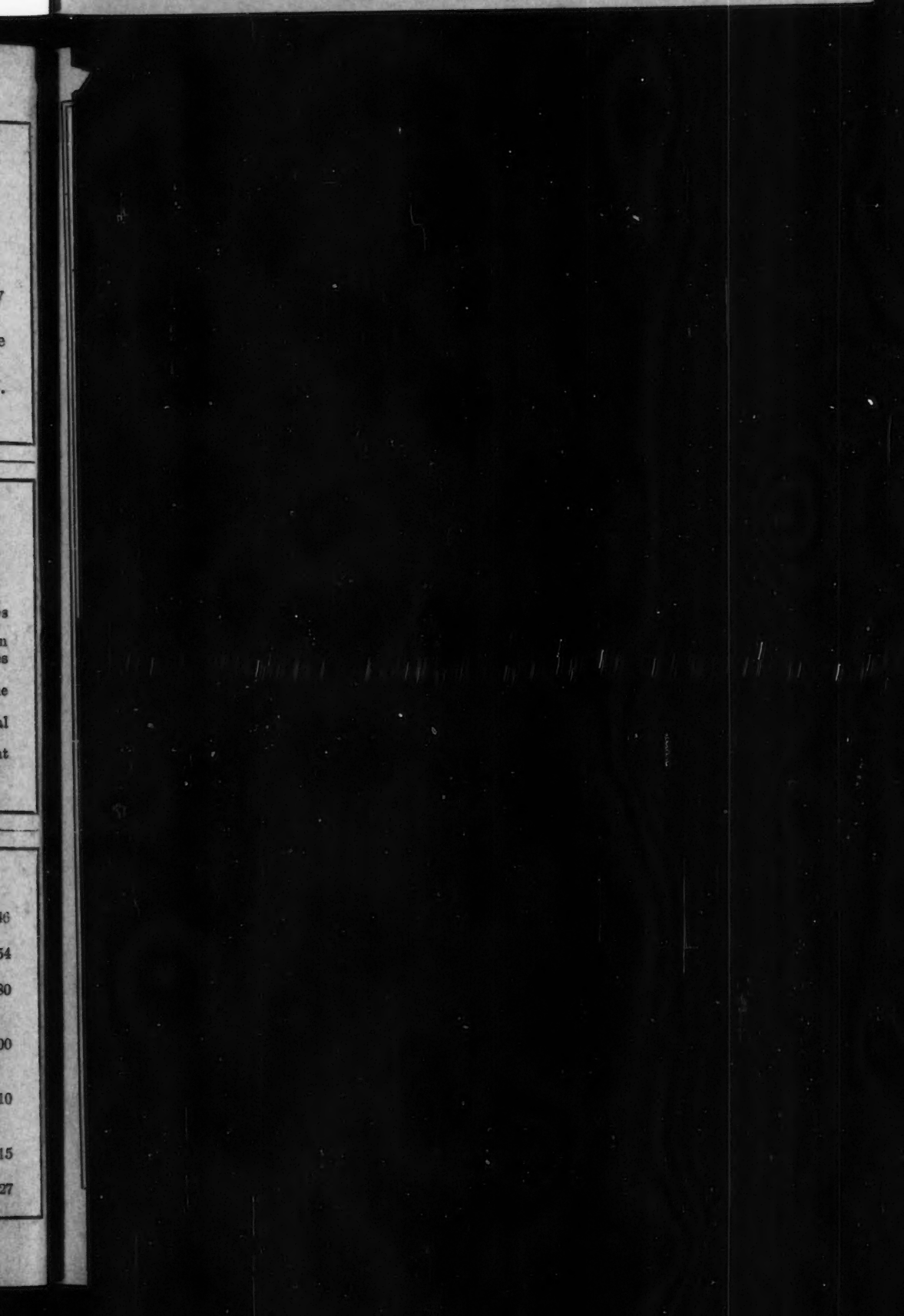
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